Charles University in Prague
Faculty of Science

Study programme: Biology
Branch of study: Cellular and Developmental Biology

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REGULACE SIGNÁLNÍ DRÁHY ERK PROSTŘEDNICTVÍM SCAFFOLD PROTEINU RACK1

THE REGULATION OF THE ERK SIGNALLING PATHWAY BY SCAFFOLD PROTEIN RACK1

Diploma thesis

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Prague 2012

Declaration

I hereby declare that I have written this diploma thesis independently under supervision of Ing. Tomáš Vomastek, Ph.D. and with the use of literature that I have appropriately cited. This work or any significant part of it has not been submitted to earn an academic degree of the same or any other kind.

In Prague 05/05/2012 .....

Bc. Vojtěch Bráborec

Acknowledgements

I sincerely thank to my supervisor Ing. Tomáš Vomastek, Ph.D. for his invaluable consultations, advices and experiences he has shared with me during the whole project. I would also like to thank to all my colleagues from the Laboratory of Cell Signalling, especially to Zuzka Sušická, M. Sc., because she introduced me into the mysteries of the laboratory work and guided me during the beginning of my project. I also thank to Míla Maninová, M. Sc. and Pepa Čáslavský, M. Sc. for their support, advices and friendly atmosphere in the lab, which helped me to achieve my goals.

Abstract

The ERK signalling cascade comprised of protein kinases Raf, MEK and ERK is an evolutionarily conserved member of MAPK family that is activated in response to wide range of extracellular stimuli. The ERK pathway controls fundamental cellular functions including cell proliferation, differentiation, apoptosis or cell motility. To control such a diverse cellular responses by a single pathway cells have evolved regulatory mechanisms that channel the extracellular signals towards the specific biological response. Crucial to this control are non-enzymatic proteins termed scaffolds that associate with and enhance functional interaction of the components of MAPK pathways and can regulate amplitude, timing, specificity and location of signals. Scaffold protein RACK1 associates with several components of cell migration machinery including integrins, FAK, Src and the ERK pathway core protein kinases. RACK1 regulates distinct steps of cell migration such as establishment of cell polarity and focal adhesion turnover, however, the molecular mechanism by which RACK1 regulates these processes remains largely unknown.

The main aim of this study was to investigate the functional role of RACK1 in cell motility, in particular to identify new effector proteins utilized by the ERK pathway and RACK1 in the regulation of focal adhesion disassembly. We observed that silencing of RACK1 impaired cell protrusivity and focal adhesion disassembly. We identified protein kinase RSK as a downstream effector of the ERK pathway that regulates focal adhesion dynamics and cell migration. Further analysis revealed that RACK1 is necessary for efficient RSK activation and, in addition, that RACK1 forms complex with RSK. Taken together, these data show that RACK1 specifically associates with ERK and RSK and consequently facilitates the RSK activation by ERK. RSK is an effector protein kinase that upon activation controls focal adhesion turnover and cell migration.

Key words:

MAPK, ERK, RACK1, RSK, adaptor protein, scaffold protein, focal adhesion, signal specificity, cell motility

Abstrakt

Signální dráha ERK, tvořená proteinkinázami Raf, MEK a ERK, je nedílnou součástí evolučně konzervovaných signálních drah MAPK rodiny umožňující eukaryotním buňkám zaznamenávat a vyhodnocovat širokou škálu vnějších podnětů. Dráha ERK převádí extracelulární signály v celou řadu specifických buněčných procesů jako je proliferace, diferenciace, apoptóza či migrace. Pro kontrolu takového množství buněčných odpovědí jedinou signální dráhou buňky vyvinuly regulační mechanizmy, které směřují signál k specifické buněčné odpovědi. Klíčovým mechanizmem regulace signální specificity jsou proteiny s neenzymatickou funkcí tzv. scaffold proteiny. Tyto proteiny váží jednotlivé komponenty MAPK dráhy a tím umožňují jejich funkční interakci, čímž určují sílu, načasování, specificitu a lokalizaci příslušného signálu v rámci buňky. Je známo, že scaffold protein RACK1 interaguje s řadou proteinů buněčné migrace, jako jsou integriny, FAK, Src a vlastní komponenty signální dráhy ERK. RACK1 také reguluje jednotlivé kroky buněčné migrace jako je ustanovení buněčné polarity a rozpad fokálních adhezí. Molekulární mechanismy stojící za těmito procesy však zůstávají nadále nejasné.

Hlavním cílem této diplomové práce bylo prostudovat funkční úlohu proteinu RACK1 v rámci buněčné migrace, především pak určit nové efektorové proteiny signální dráhy ERK a proteinu RACK1, které se podílejí na regulaci rozpadu fokálních adhezí. Zjistili jsme, že snížení exprese RACK1 vede k narušení buněčné protruzivity a poruše rozpadu fokálních adhezí. Identifikovali jsme proteinkinázu RSK jako efektorový protein dráhy ERK, který reguluje fokální adheze a buněčnou migraci. Další experimenty odhalily, že protein RACK1 je nezbytný pro řádnou aktivaci proteinkinázy RSK a navíc s ní vytváří proteinový komplex. V souhrnu naše výsledky prokazují, že RACK1 specificky váže proteinkinázy ERK a RSK, čímž usnadňuje aktivaci RSK pomocí proteinkinázy ERK. RSK následně funguje jako efektorový protein, který, je-li aktivován, reguluje rozpad fokálních adhezí a tím i buněčný pohyb.

Klíčová slova:

MAPK, ERK, RACK1, RSK, adaptorový protein, scaffold protein, fokální adheze, signální specifita, buněčná migrace,

Content

List of abbreviations

AP-1	activator protein 1
CD	common docking
CDK	cyclin-dependent kinase
CR	conserved region
CRD	cysteine-rich domain
CTKD	C-terminal kinase domain
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
GAP	GTPase accelerating protein
GEF	guanine nucleotide exchanging factor
GPCR	G protein-coupled receptor
GSK3	glycogen synthase kinase 3
Gβ	G protein subunit β
KD	knockdown
MAPK	mitogen-activated protein kinase
MAPKAPK	MAPK activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MLCK	myosin light chain kinase
MP1	MEK partner 1
MT	microtubule
NTKD	N-terminal kinase domain
PAK	p21 Rac-activated kinase

PDK1	3-phosphoinositide dependent protein kinase-1
PEA-15	15kDa phosphoprotein enriched in astrocytes
PKC	protein kinase C
PP2A	protein phosphatase 2A
RACK1	receptor for activated protein kinase C
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma
RBD	Ras binding domain
RKIP	Raf kinase inhibitor protein
ROCK	Rho-associated, coiled-coil containing protein kinase 1
RSK	ribosomal S6 protein kinase
RTK	receptor tyrosine kinase
SH2	Src-homology 2
VASP	vasodilator-stimulated phosphoprotein
WAVE	WASP family Verprolin-homologous protein
WRC	WAVE2 regulatory complex

1. Introduction

Cells exist in constantly changing environment to which they have to adapt in order to survive. The changes of the environment are carried in form of diverse extracellular stimuli that are received and processed by the cells and that trigger distinct cellular functions. One of the most efficient mechanisms to integrate broad range of inputs that cells developed are signalling pathways. Among the most ancient groups of signalling pathways utilized in various physiological processes by majority of organisms are mitogen-activated protein kinase (MAPK) pathways. This study is focused on one particular member of this group – the extracellular signal-regulated kinase (ERK) pathway. The ERK pathway is the most intensively studied and so far the best described member of the MAPK family that consists of three core protein kinases – Raf (rapidly accelerated fibrosarcoma), MEK (MAPK/ERK kinase) and ERK. The core protein kinases sequentially phosphorylate and thus activate each other, which leads to rapid conversion of extracellular signals to relevant biological outcomes such as cell proliferation, differentiation, cell cycle control, apoptosis, cell survival or regulation of motility.

The importance of fully functional and unperturbed ERK signalling is best demonstrated by various embryonic defects including malfunctioning nervous or cardiovascular system caused by defective components of the signalling cascade. However, not only prenatal development is endangered by the impeded signal transmission. Constitutive activation of the ERK signalling triggered by somatic mutations is the main culprit of many types of human cancer. Since the beginning of the cancer research, new potential targets that are either members of the ERK pathway, or closely related proteins have been selected. Thus, understanding of the events mediated by the ERK signalling is not only interesting, but it is and will be crucial for the future developments in the cancer research.

Despite the important role of the ERK signalling cascade in many cellular functions it is still quite unclear how exactly the multitude of extracellular signals is implemented into specific biological responses. That is why great deal of attention has been paid to address this question in recent years. Several molecular mechanisms have emerged with so called adaptor proteins being highlighted as signal specificity master regulators. It is being more and more apparent that the adaptor proteins, in addition to

core protein kinases of the ERK pathway, can dramatically alter the biological response and therefore function as important contributors to signal specificity determination.

RACK1 (receptor for activated protein kinase C) is such an adaptor protein with scaffolding function that is required for efficient ERK signalling in response to cell adhesion. Despite numerous works, we still have only limited information of how RACK1 modulates the chain of events downstream of the ERK pathway. The central concept of this work, upon which the goals are built, is that RACK1 integrates signals coming from various sources upstream of the ERK pathway, mediates the activation of the ERK signalling module and links it to downstream effectors. Therefore, this study is focused on investigating the exact role of the scaffold protein RACK1 in the ERK-mediated regulation of cell motility. We concentrated on identifying new potential substrates of ERK that are involved in the cell migration process and that require the RACK1 scaffolding function to efficiently associate with ERK.

2. Literature review

2.1. The basic architecture of the MAPK cascades

The typical MAP kinase pathway displays an evolutionary conserved three tier architecture consisting of three protein kinases: a mitogen–activated protein kinase (MAPK), a MAP kinase kinase (MAPKK) and a MAP kinase kinase kinase (MAPKKK). These three kinases form a central signalling unit that transmits signals by sequential phosphorylation and activation from MAPKKK to MAPKK and to MAPK. This arrangement provides many advantages such as fast and efficient signal transduction and amplification on its way from extracellular space to its final location. The three tier architecture is also essential for signal specificity determination and tight regulation of transmitted signals as is allows the cells to alter their behaviour or even a cellular programme according to the situation (for example to switch from proliferation to differentiation).

According to their architecture, the MAPK pathways can be divided into two groups - conventional and atypical. The conventional MAPK signalling cascades share typical MAPKKK-MAPKK-MAPK three tier architecture. In higher eukaryotes four conventional MAPK signalling cascades have been identified and they comprise of ERK, JNK, p38 and ERK5 pathways. ERK3/4 and ERK7 are two examples of the

atypical MAPK pathways. The atypical MAPK signalling cascades do not have the three tier architecture and they are categorized as MAPKs due to their sequence homology with ERKs (Cargnello and Roux, 2011).

2.2. The ERK signalling cascade – properties and regulation of signalling specificity

2.2.1. The activation of the ERK pathway

The ERK cascade is the most intensively studied and so far the best described member of the MAPK family that consists of three core protein kinases – Raf, MEK and ERK.

The ERK signalling pathway is activated by various extracellular agents that bind to receptors localized to plasma membrane (Figure 2.1). The activators include growth factors, hormones, neurotransmitters or cytokines and they act through receptor tyrosine kinases (RTKs), G-protein-coupled receptors (GPCRs) or integrins, proteins interacting with extracellular matrix. The ERK pathway can also be activated to a various extent by osmotic, genotoxic and other cellular stresses or by depolymerization of microtubules (Cargnello and Roux, 2011).

The activation of the ERK pathway is best described on signalling through EGF (epidermal growth factor) and EGFR (EGF receptor) (Figure 2.1). EGFR is RTK type of receptor, which upon binding of EGF undergoes dimerization that induces its tyrosine kinase activity. Activated EGFR subsequently autophosphorylates on tyrosine residues within its cytoplasmic domains creating docking sites for SH2 (Src homology 2) domain of adaptor protein Grb2. Grb2 exists in complex with guanine nucleotide exchange factor SOS. Upon EGFR activation, the Grb2-SOS complex translocates from cytoplasm to plasma membrane and brings SOS into close proximity with small GTPase Ras. Protein SOS subsequently activates Ras (Cargnello and Roux, 2011).

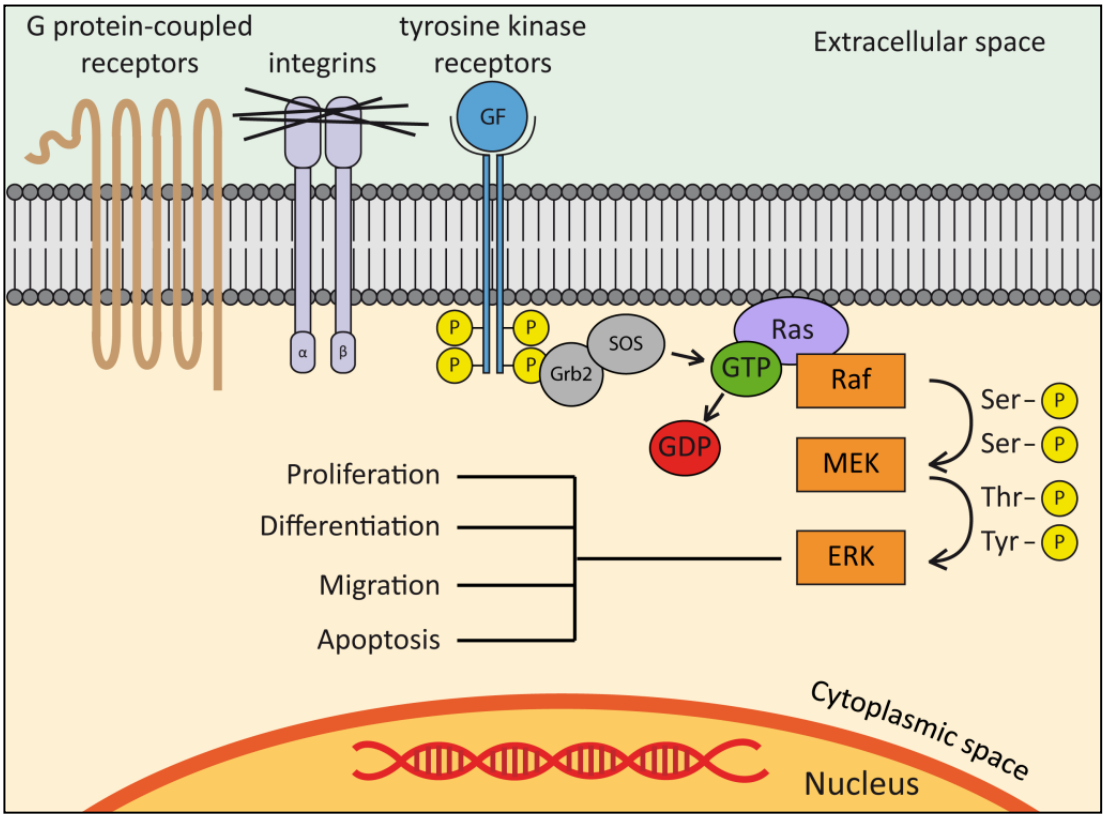

Figure 2.1 – Schematic representation of the ERK pathway activation, signal transduction and biological outcome. The ERK pathway is activated in response to various stimuli binding to their respective receptors, including receptor tyrosine kinases, G protein-coupled receptors and integrins. Once activated, receptors transmit the signal towards small GTPase Ras. When acting through the tyrosine kinase receptors, the signal is transmitted by adaptor protein Grb2 and SOS, a guanine nucleotide exchange factor for Ras. Active Ras facilitates relocalization of a protein kinase Raf from cytoplasm to plasma membrane. On the plasma membrane Raf undergoes series of phosphorylation events triggering its activation. Active Raf phosphorylates protein kinase MEK thus ensuring its stimulation. MEK is a dual specificity protein kinase that phosphorylates and therefore activates protein kinase ERK. Activated ERK further propagates the signal by changing phosphorylation status of numerous targets localized in cytoplasm and in nucleus, which subsequently leads to diverse biological responses like proliferation, differentiation, cell migration or apoptosis. Adapted and modified from (Brown and Sacks, 2009).

2.2.2. Small GTPase Ras

The activation of Ras is a crucial step in the signal transduction across plasma membrane as active Ras is involved in several signalling pathways, including the ERK

pathway (Figure 2.1). Ras proteins are localized predominantly to plasma membrane. The membrane localization is promoted by posttranslational modifications such as myristoylation and/or prenylation (Clarke, 1992).

Ras is a member of a family of small GTPases. The small GTPases cycle between two basic conformational states. Ras is activated when bound to GTP and inactive when bound to GDP. These two states are regulated by intrinsic GTPase activity of Ras that is modulated by association with inactivating proteins that increase GTP hydrolysis (GTPase activating proteins, GAPs) and activating proteins that promote the exchange of GDP for GTP (guanine nucleotide exchange factors, GEFs). This mode of regulation allows rapid switch between both states in on/off manner (Rajalingam et al., 2007).

Mammalian genomes encode three *ras* genes that give rise to four protein products – N-Ras, H-Ras, K-RasA and K-RasB. All three genes are expressed ubiquitously to extent, which is tissue-specific. *Ras* genes are oncogenes that have great impact on human health. About 30 % of all human cancers are caused by constitutively active Ras proteins. The constitutive activation of Ras usually arises from mutations that lead to insensitivity to GAPs (Mor and Philips, 2006).

Once activated, the GTP-bound form of Ras binds directly to Raf and recruits it to plasma membrane. The membrane localization subsequently leads to activation of Raf, a complex step during the signal transduction through the Raf-MEK-ERK signalling module.

2.2.3. Core components of the ERK pathway

2.2.3.1. Raf

Raf is a serine/threonine protein kinase that facilitates signal transduction from the plasma membrane to protein kinases MEK1 and MEK2.

There are three known mammalian Raf isoforms – A-Raf, B-Raf and C-Raf (also termed Raf-1 or c-Raf-1) (Figure 2.2). All isoforms are ubiquitously expressed throughout most tissues. Although individual isoforms are to some extent functionally redundant, genetic studies in mice have shown that Raf proteins carry out non-redundant functions in development (Wellbrock et al., 2004). Although C-Raf has been the first isoform discovered and so far the best characterized, B-Raf has in recent years

come into spotlight for its clinical significance. It was discovered that the constitutive activation of B-Raf is the main cause of many types of human cancer. For example, B-Raf mutations have been detected in 70 % of all melanomas, 30 % of thyroid cancers or 15 % of colon cancers. The majority of the mutations occur within the kinase domain (Wan et al., 2004). V600E substitution alone accounts for 90 % of the mutations. The substitution of valine by glutamic acid leads to insertion of negative charge, which is an effect comparable to phosphorylation of amino acid and that is the reason why B-Raf remains constitutively active (Davies et al., 2002).

Each of the Raf kinases shares three CR (conserved regions) – CR1, CR2 and CR3 (Figure 2.2). CR1 consists of Ras-binding domain (RBD) and cysteine-rich domain (CRD). Through CR1 Raf interacts with Ras and with membrane phospholipids. CR2 is a serine/threonine-rich domain that contains amino acid (Ser259 in C-Raf) that, when phosphorylated, serves as a docking site for 14-3-3 inhibitory protein. CR3 is a C-terminal kinase domain that contains second 14-3-3 docking site (Ser582 in A-Raf, Ser729 in B-Raf and Ser621 in C-Raf) (Roskoski, 2010).

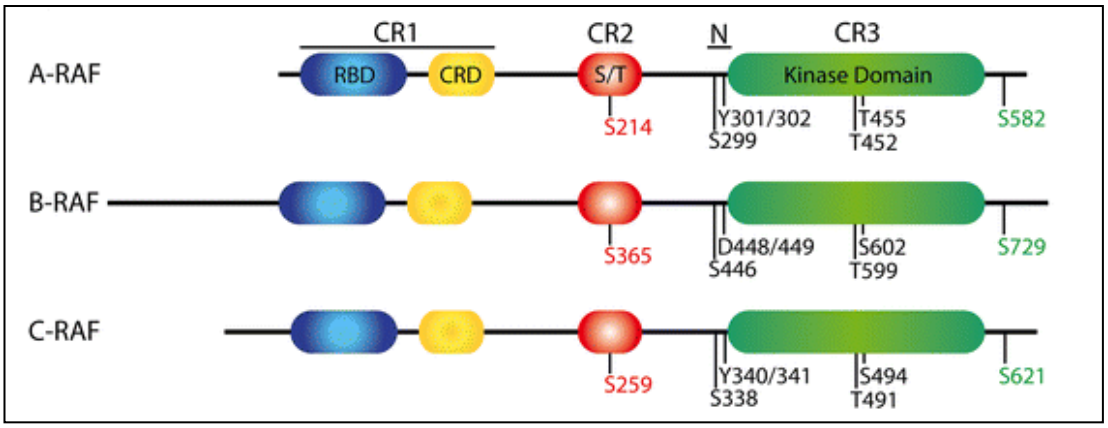

Figure 2.2 – The domain organization of different Raf isoforms. All three Raf isoforms share three conserved regions (CR 1-3). CR1 consists of two functionaly different domains – RBD (Ras binding domain), responsible for binding of Ras and CRD (cysteine-rich domain), responsible for binding of membrane lipids. CR2 is a serine/threonine rich domain that associates with 14-3-3 inhibitory protein. CR3 is a kinase domain that is also involved in the binding of 14-3-3. Below each isoform are indicated conserved phosphorylation sites. In red and green color are depicted amino acids that are bound by the 14-3-3 proteins (Udell et al., 2011).

C-Raf has been so far the most extensively studied of all three isoforms with the best described mode of regulation. When inactive, the catalytic CR3 domain is folded

and bound to the N-terminal regulatory domain. Binding of 14-3-3 proteins at the two amino acid residues stabilizes this interaction. Upon stimulation, active Ras-GTP binds to CR1 and disrupts the interaction of 14-3-3 with Ser259. This leads to partial unfolding of Raf. Although the binding of Ras is necessary for the Raf activation, it is not sufficient to fully activate Raf (Udell et al., 2011). To get fully activated, other modifications mediated by membrane localized signalling proteins have to be involved. These include dephosphorylation of Ser43, a negative regulatory site targeted by PKA, Ser338/339 and Tyr340/341, sites targeted by PAK (p21 Rac-activated kinase) and Src family of protein kinases, respectively. All these phosphorylations lead to weakening of the inhibitory effect of the N-terminal part and to stabilization of the active conformation of C-Raf (Leicht et al., 2007). Although activated Raf can phosphorylate several proteins, the phosphorylation and activation of protein kinase MEK is dominant and the most important Raf function.

2.2.3.2. MEK

MEK1 and MEK2 (further referred to as MEKs) are dual specificity protein kinases that are activated by phosphorylation of two serine residues (Ser218, Ser222 in MEK1 and Ser222, Ser226 in MEK2) at typical Ser-Xxx-Ala-Xxx-Ser motif in their activation loop (Alessi et al., 1994). While Raf is the principal activator of MEKs, there are several proteins functioning at the level of MAPKKs. Under some circumstances (in specific cell type or when stimulated by specific agent) protein kinases Mos or Tpl2 are utilized instead of Raf (Raman et al., 2007).

Besides the phosphorylations in the activation loop of MEKs, there are other additional regulatory sites that undergo posttranslational modifications. Phosphorylation of MEK1 on Ser298 by PAK1 is required for an enhancement of MEK1-ERK interaction. PAK1 ability to recognize and phosphorylate Ser298 can be disrupted by ERK-mediated phosphorylation of Thr292. This process is stimulated by cell adhesion and provides a negative feedback mechanism by which active ERK can regulate the PAK1 phosphorylation of MEK1 (Eblen et al., 2004).

MEKs belong to small group of dual specificity protein kinases, proteins with ability to recognize and phosphorylate both Thr and Tyr residues. MEKs display very narrow substrate selectivity towards native form of ERK. Protein kinases ERK are the only known MEK substrates (Roskoski, 2012).

2.2.3.3. ERK

Protein kinases ERK (further referred to as ERKs) belong to the most extensively studied group of MAP kinases. ERKs are encoded by two distinct genes, which give rise to several protein products – ERK1, ERK2 and three alternatively spliced forms, ERK1b (Yung et al., 2000), ERK1c (Aebersold et al., 2004) and ERK2b (Gonzalez et al., 1992).

ERKs carry a characteristic signature motif Thr-Glu-Tyr (TEY) in the activation loop. Inactive MEKs bind to ERKs and retain them in cytoplasm (Fukuda et al., 1997). When stimulated, MEKs phosphorylate the TEY motif at Thr and Tyr (Thr202, Tyr204 in ERK1 and Thr183, Tyr185 in ERK2) which results in full activation of ERKs and their dissociation from MEKs (Haystead et al., 1992).

ERKs are proline-directed protein kinases that recognize and phosphorylate serine or threonine residue in a consensus sequence Pro-Xxx-Ser/Thr-Pro (Rubinfeld and Seger, 2005). However, the presence of the first proline is not always required. Because of the quite broad nature of the substrate recognition motif, the protein kinase ERK, in contrary to the ERK upstream signalling components MEK and Raf, functions as an effector protein kinase. Almost two hundred proteins have been identified as ERK substrates including cytoskeletal proteins, protein kinases and transcription factors (Yoon and Seger, 2006).

To interact with MEKs or various substrates, ERKs utilize so called docking interactions. The docking interactions mediate association with the ERK substrates but apart from transient enzyme-substrate interactions, they take place at different portion of protein, not in the active centre (Tanoue and Nishida, 2003). ERKs utilize two types of docking regions – a CD (common docking) domain and an ED site. Both regions form a docking groove which enhances the binding of the ERK substrates to ERKs (Tanoue et al., 2001). The CD domain contains a cluster of negatively charged amino acids that are recognized by positively charged region in the ERK-interacting molecule called D-domain (or DEJL motif). The D-domain was found not only in the ERK substrates, but also in MEKs and MKPs (Tanoue et al., 2000). Many ERK substrates possess a second docking motif – DEF (also known as Phe-Xxx-Phe-Pro or FXFP) (Fantz et al., 2001). However, the DEF motif does not appear in all ERK-interacting proteins suggesting that it increases specificity of ERKs only to some specific targets. Thus, the

presence of the ERK interaction motif and the phosphorylation consensus sequence are necessary for efficient substrate recognition and phosphorylation by ERKs.

2.2.4. ERK substrates and signalling downstream of ERK

It is important to understand that the ERK pathway functions as a key signalling node that integrates several inputs and turns them into plethora of cellular outputs. Thus, the important question concerning the ERK signalling is how a single protein kinase can generate such a variety of different responses such as cell proliferation or differentiation. Current concept is that the specific cellular response is achieved through control of activity of wide range of ERK substrates that are available for phosphorylation by ERK at a given moment in a particular location. Up to date, almost two hundred different protein targets of ERK have been discovered (Yoon and Seger, 2006). Thus, the selection and phosphorylation of specific ERK substrates is one of the most important factors determining the biological outcome.

Various proteins have been identified as the ERK substrates including cytoskeletal proteins, protein kinases and transcription factors. However, in very few cases the link between the substrate phosphorylation and specific biological outcome has been demonstrated.

The best described is a role of ERK in the regulation of transcription through the phosphorylation of Elk1 and c-Fos transcription factors. Elk1 is a member of a ternary complex factor subfamily of ETS-domain transcription factors which, upon the ERK-mediated activation, regulates transcription of c-Fos by binding to serum response element and by interacting with serum response factor. c-Fos together with c-Jun forms AP-1 (activator protein 1), an important regulator of expression of immediate early genes such as c-Myc. AP-1 is also required for expression of cyclin D1, a protein that interacts with CDKs (cyclin-dependent kinases) and promotes G_1/S transition and cell cycle progression (Shaulian and Karin, 2001).

In addition to transcription factors, protein kinases ERK also regulate function of many cytoplasmic proteins including cytoskeletal proteins or transmembrane receptors. Recently, the activation of MAPKAPKs (MAPK activated protein kinases), protein kinases functioning downstream of MAPK, emerged as a common theme in signalling by the typical MAPK cascades. All MAPKAPKs share similar structure, mode of activation and they recognize the same phosphorylation motif (Cargnello and Roux,

2011). The p90 ribosomal S6 kinase (RSK) is activated specifically by ERKs and it is an intensively studied member of the MAPKAPK group, which is involved in regulation of many physiological processes including cell migration. Similarly to ERKs, the protein kinase RSK is rather a promiscuous protein kinase as it phosphorylates and thus alters function of dozens of proteins (Anjum and Blenis, 2008).

2.2.4.1. The regulation and function of protein kinase RSK

RSK is a 90kDa Ser/Thr protein kinase that belongs to a family consisting of four human isoforms - RSK1-4. All RSK isoforms (further referred to as RSKs) share high degree of sequential identity (75-80 % amino acid identity) and are uniquely characterized by presence of two functionally distinct kinase domains (Fisher and Blenis, 1996). While RSK1-3 are expressed ubiquitously in all human tissues and they seem to be, at least to some extent, functionally redundant (Zeniou et al., 2002), the overall RSK4 expression is much lower and it was detected only in brain, heart and kidney tissue (Dummler et al., 2005). RSKs recognize and phosphorylate many cytosolic and nuclear targets and they are therefore involved in regulation of various physiological processes including protein translation, cell proliferation, survival or motility (Anjum and Blenis, 2008).

RSK proteins have structure unique among other protein kinases - RSKs are comprised of two catalytically active kinase domains joined together by a linker region. NTKD (N-terminal kinase domain) shares homology with kinases of AGC family. CTKD (C-terminal kinase domain) is homologous to calcium/calmodulin-dependent protein kinases. While the CTKD is responsible for autophosphorylation and via its D-domain facilitates binding of ERKs, the NTKD is required for phosphorylation of RSK substrates (Fisher and Blenis, 1996).

RSKs are phosphorylated and thus activated by ERKs in response to various extracellular stimuli including growth factors, hormones, neurotransmitters and other stimulating agents. The activation of RSKs is a complex process that requires cooperation of both kinase domains and additional phosphorylation of multiple amino acids mediated by protein kinases ERK and PDK1 (3'-phosphoinositide-dependent kinase-1) (Figure 2.3). Upon mitogen stimulation, ERKs phosphorylate Thr359 and Ser363 localized in the linker region and Thr573 in the CTKD activation loop which leads to activation of the CTKD. When active, the CTKD phosphorylates Ser380 in a

hydrophobic motif creating a docking site for PDK1. After binding, PDK1 phosphorylates Ser221 in the NTKD activation loop rendering a fully active RSK. The NTKD also phosphorylates Ser749 in the D domain and thereby modulates the binding of ERK (Anjum and Blenis, 2008).

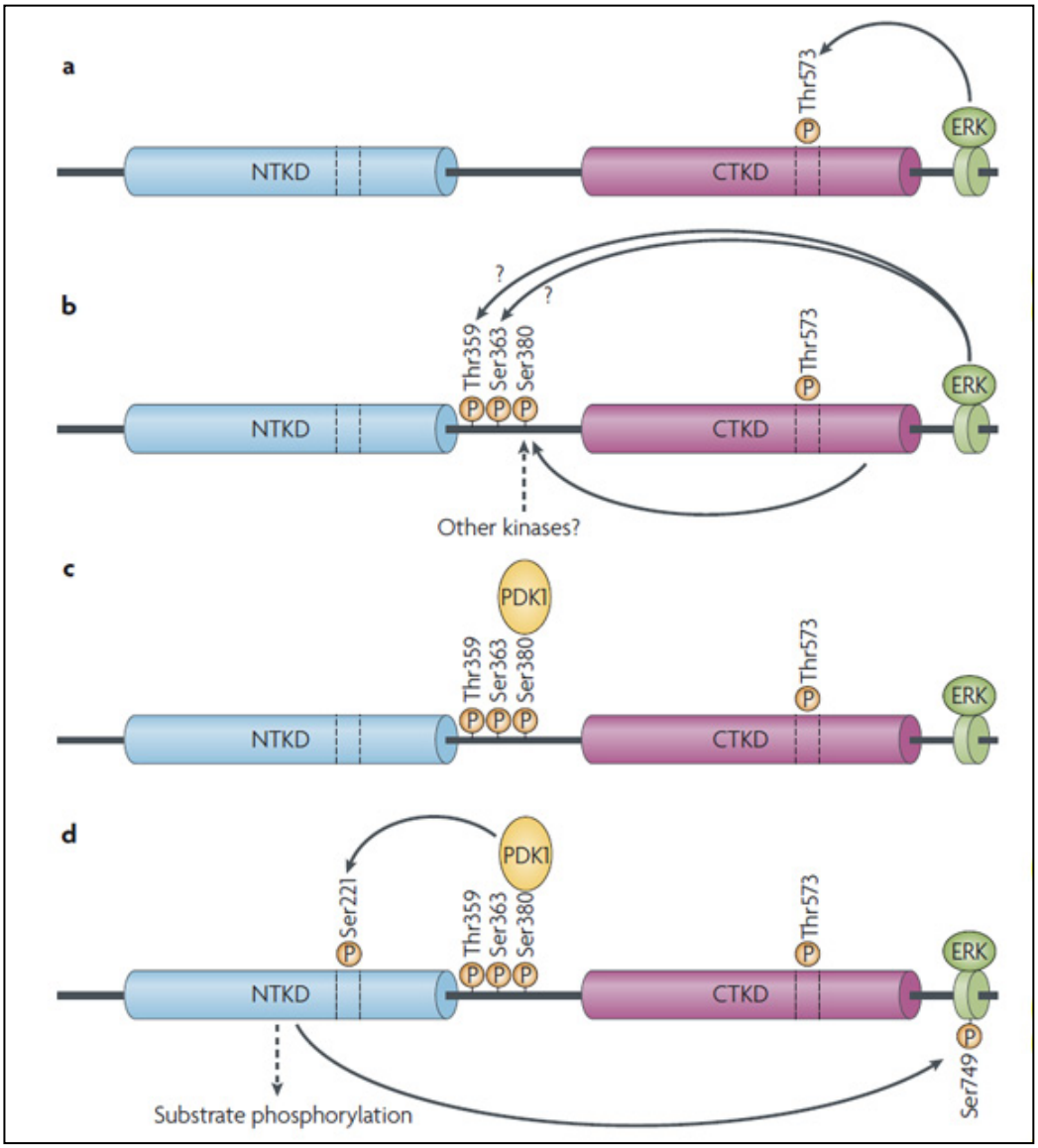

Figure 2.3 – Current model of RSK activation. To be fully activated, protein kinase RSK requires phosphorylation of several amino acid residues mediated by ERKs and PDK1. Model adapted from (Anjum and Blenis, 2008).

As the name "p90 ribosomal S6 kinase" implies, RSKs have been identified as protein kinases phosphorylating the ribosomal protein S6 (Erikson and Maller, 1985). Following studies revealed that RSKs regulate protein translation by phosphorylation of

multiple ribosome-associated proteins (Frodin and Gammeltoft, 1999). In addition to ribosomal protein S6, RSKs phosphorylate Tuberous sclerosis complex-2 at Ser1798, inactivate it and promote mTOR signalling and protein translation. RSKs also directly affect the translational machinery by phosphorylating eukaryotic translation initiation factor-4B (Anjum and Blenis, 2008).

Activation of RSKs results in increased nuclear accumulation of RSKs. In the nucleus, RSKs regulate transcription by controlling the activity of transcription factors CREB, SRF, NF-κB or ERα. RSKs also phosphorylate number of immediate early genes, including c-Fos or c-Jun or transcriptional co-activators CBP and p300 (Anjum and Blenis, 2008). RSKs also affect expression of many genes through phosphorylation and inhibition of GSK3 (glycogen synthase kinase-3). GSK3 phosphorylates cyclin D2 and c-Myc which results in degradation of these proteins. GSK3 also modifies c-Jun which prevents it from binding to DNA (Frame and Cohen, 2001).

Among other functions, RSKs also regulate cell migration although the molecular mechanism remains poorly understood. RSK binds filamin A, a component of cytoskeletal machinery that crosslinks actin filaments and promotes efficient cell migration. RSK facilitates filamin A phosphorylation on Ser2152 (Woo et al., 2004) which was shown to be facilitated by PAK1 and required for membrane ruffling (Vadlamudi et al., 2002).

2.2.5. Shaping the ERK cascade by adaptor proteins

Protein inhibitors, anchoring proteins and scaffold proteins (here collectively termed as adaptor proteins) represent an efficient regulatory mechanism that allows cells to dynamically shape the signal transduction towards the desired outcome. Adaptor proteins are predominantly non-enzymatic proteins that physically associate with one or more components of the ERK cascade. As their designation implies, they can modulate the signalling by the ERK pathway by inhibiting the signal transduction (protein inhibitors), bringing the signalling components to close proximity (protein scaffolds) or by regulating the localization of the ERK cascade (protein anchors) (Figure 2.4). However, this classification is not strict as many adaptor proteins possess more than one function and therefore can belong to more groups.

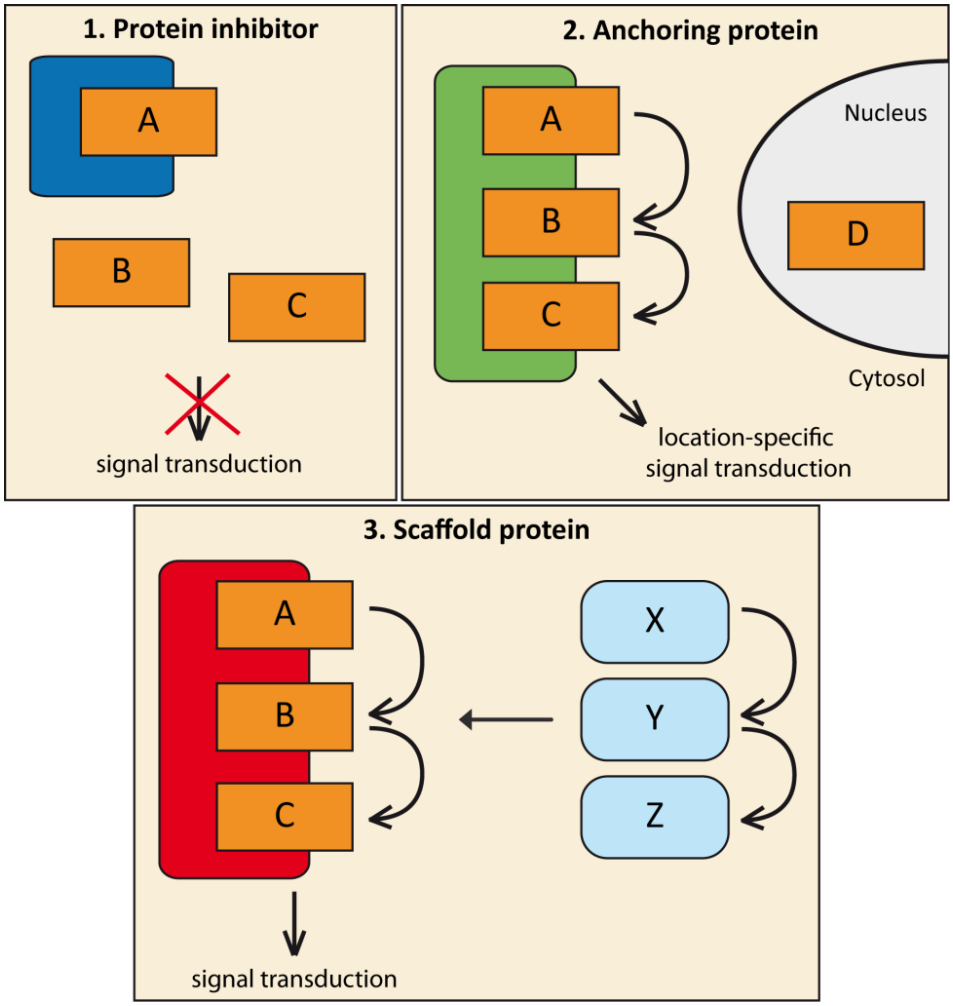

Figure 2.4 – The functions of adaptor proteins. Adaptor proteins may regulate the ERK signalling pathway by three basic mechanisms. (1) Protein inhibitors block the signal transduction by preventing a physical interaction and therefore activation of signalling components. (2) Anchoring proteins facilitate transmission of signals in a site- or compartment-specific manner. (3) Scaffold proteins bind several signalling components, bring them into close proximity and thereby facilitate signal transduction. In addition, scaffold proteins often integrate signals from parallel pathways to modulate the activation of the ERK cascade.

2.2.5.1. Protein inhibitors

Protein inhibitors bind one or two components of the signalling cascade and prevent their interaction, thereby disrupting the signal propagation between the proteins. The importance of the protein inhibitors is underlined by the fact that in numerous cancers the expression of the protein inhibitors such as RKIP (Raf kinase inhibitor protein) is significantly attenuated suggesting that they play an important role in the

carcinogenesis (Fu et al., 2003; Schuierer et al., 2006). RKIP associates with Raf and prevents its physical interaction with MEKs. As a consequence, Raf is unable to activate MEKs (Yeung et al., 1999). Similarly, protein inhibitor Sprouty prevents activation of Ras by blocking its association with Grb-2 (Gross et al., 2001) and inhibits the Ras-mediated Raf activation (Sasaki et al., 2003).

2.2.5.2. Anchoring proteins

Protein anchors modulate activity of the signalling components of the ERK pathway by capturing them in specific cellular locations, thereby enhancing a phosphorylation of a specific set of downstream substrates. Or taken from another perspective, the anchoring proteins prevent the phosphorylation of a specific set of substrates by retaining them in a specific subcellular location.

PEA-15 (15kDa phosphoprotein enriched in astrocytes) is an example of the typical anchoring protein without any enzymatic functions. PEA-15 can retain ERKs in cytoplasm (Formstecher et al., 2001). PEA-15 anchoring function arises from its ability to mediate a nuclear export via a nuclear export sequence and to prevent the ERK translocation into nucleus by blocking ERK binding to nuclear pores (Whitehurst et al., 2004). Even the integral components of the ERK pathway can function as the anchoring proteins. For example, MEKs anchor inactive ERKs in the cytoplasm, thus making a notable exception from the fact that the adaptor proteins do not possess any enzymatic activity (Ramos, 2008).

2.2.5.3. Scaffold proteins

Scaffolds together with other adaptor proteins play crucial role in determination of biological outcome because they regulate the signal specificity in many ways. Originally identified in yeasts, these non-enzymatic proteins bind simultaneously the signalling components of the MAPK cascade, bring them into close proximity and facilitate their functional interaction that results in enhanced signal transduction. The scaffolds exclude non-relevant proteins, thus preventing a crosstalk with other signalling pathways and, similarly to the anchoring proteins, define specific subcellular localization of many signalling components. Since scaffolds also associate with upstream activators and downstream targets of the MAPK pathway, they function as

signalling channels that rapidly and specifically transmit extracellular signals (Schwartz and Madhani, 2004).

Several scaffold proteins have been identified in higher eukaryotes. KSR is the best studied scaffold of the ERK pathway and it is functionally most similar to yeast scaffolds. KSR interacts with the components of the ERK pathway and forms a Raf-MEK-ERK multiprotein complex. Formation of the multiprotein complex is a prerequisite for the efficient ERK activation by the growth factor receptors and growth factors-induced mitogenesis (Nguyen et al., 2002). Similarly, RACK1 was shown to interact with all three core protein kinases of the ERK signalling pathway, to tether them and thereby facilitate their activation upon specific signal, the cell adhesion (Vomastek et al., 2007).

The mammalian scaffolds also display characteristic properties not seen in yeasts. Some scaffold proteins like MP1 (MEK partner 1) are able to integrate signals from parallel pathways. MP1 and its partner p14 regulate the ERK activation during cell adhesion and cell spreading. MP1 interacts specifically with MEK1 and ERK1 as well as with protein kinase PAK1 (Pullikuth et al., 2005). Upon association with MP1, PAK1 facilitates phosphorylation of MEK1 serine residue 298. This phosphorylation increases the association of MEK1 with ERK1 and thereby promotes signalling along the ERK pathway (Eblen et al., 2004).

The scaffolds can, similarly to the anchors, regulate localization of ERK to specific cellular compartments. The scaffold proteins RACK1, paxillin and GIT1 have been shown to regulate the ERK localization to focal adhesions, although the reason for such functional redundancy is unclear.

The above referenced studies suggest that the activation of the ERK pathway results in the phosphorylation of hundreds of different proteins. These phosphorylations are carried out by both ERKs and protein kinases downstream of ERK. Considering that the phosphorylation of the specific set of substrates is required to a bgenerate relevant biological response, this poses the central question of the ERK signalling: how do protein kinases discriminate between substrates to select the proper target? To modulate the ERK pathway toward specific biological outcomes multitude of mechanisms of the ERK regulation have evolved. The non-enzymatic components of the ERK pathway, protein scaffolds, inhibitors and anchors, emerged as important regulators that dictate the signal strength, duration, location and substrate selection within the ERK pathway.

2.3. The ERK-dependent biological functions

The functions attributed to the ERK module are diverse and can be divided into cellular and physiological level. At the cellular level, the ERK cascade contributes primarily to cell cycle progression, cytokinesis, transcription or differentiation. In addition to these key functions, the ERK module contributes to regulation of cell death, senescence, architecture of actin and microtubule cytoskeleton, cell adhesions or facilitates neurite extension and cell motility. At the physiological level, cells utilize the ERK signalling cascade to control immune system development, homeostasis and antigen activation, memory formation, heart development and hormone and growth factor response. Considering this list of diverse ERK-dependent functions it is not surprising that aberrations in the ERK module are responsible for many known pathologies including many types of cancer, diabetes, neural or cardiovascular diseases.

2.3.1. The ERK pathway in the regulation of cell motility

The ERK signalling pathway contributes to the regulation of cell migration, which is a fundamentally important process. The cell migration plays central part in wound recovery, embryonic development or immune response. It is not surprising that defects in the migration frequently result in serious consequences such as developmental failures or tumor progression and metastasis. Understanding the underlying molecular mechanisms is therefore of utmost importance, however, dissecting the signalling networks proves to be a great challenge. Despite a growing body of evidence concerning individual steps of cell migration, we still have rather limited information about coordination of individual processes. It is difficult to put the uncovered data in a context as it is clear that the regulation depends on many diverse factors including for instance composition and physical properties of extracellular matrix (ECM) or cell type.

2.3.1.1. The migration cycle

Cell migration is a multistep process that is initiated in response to extracellular signals such as gradient of mitogens or extracellular matrix proteins. Cells initiate the cell migration by polarizing and extending protrusions of plasma membrane in the

direction of the migration. The protrusion is then stabilized by formation of focal adhesions. The focal adhesions physically link the cell with the proteins of ECM and generate traction necessary for the movement. As the cell moves forward, the adhesions initially localized at the leading edge either disassemble or mature in more stable structures (Figure 2.5). The maturation of individual adhesions is accompanied by changes in protein composition and also by relative relocalization in respect to the position of the cell body. In other words, cell moves forward but the adhesions stay where they were originally created. The so called migration cycle is completed when the cell disassembles adhesions that are localized at the rear end (trailing edge) of the cell (Parsons et al., 2010).

The migration cycle is driven by remodelling of cytoskeletal network, particularly by the actin polymerization. Lamellipodium is a structure made of actin arranged in a branched (or dendritic) network that is localized beneath the plasma membrane (Figure 2.5). The polymerization of actin within the lamellipodium is the driving force of cell motility as it pushes the leading edge forward in the direction of the migration. The actin nucleation and polymerization is catalyzed by Arp2/3 complex. Activation of the Arp2/3 complex requires activity of Wiskott-Aldrich syndrome proteins WASP/WAVE which are regulated by the family of small GTPases Rac and Cdc42 (Pollard and Borisy, 2003). In addition, GTPase RhoA contributes to regulation of the leading edge by activating formin mDia1 that initiates the assembly of filamentous actin (Narumiya et al., 2009). Further beneath the plasma membrane forms a lamellum that is characteristic for actin fibres arranged in parallel bundles. The central and rear regions of the cell are interwoven with actin filaments that are typically present as thick bundles called stress fibres (Figure 2.5). The formation of the stress fibres is regulated by the GTPase RhoA (Amano et al., 1997).

Following the membrane protrusion, cell creates links with the ECM in the region of the lamellipodium - nascent adhesions. The formation of the nascent adhesions is triggered by the engagement of integrin receptors to the ECM and by clustering of the integrin heterodimers. The integrin clusters serve as a platform for binding of structural adhesion components such as paxillin, talin, vinculin or α-actinin (Lo, 2006). Structural proteins physically interact with actin fibres, thus creating the link between the ECM and the actin cytoskeleton. During the process of the adhesion maturation the adhesive structures undergo enrichment by additional protein components. This leads to distinct size and lifetimes of focal complexes and focal

adhesions (Zamir et al., 1999). In addition to structural proteins, the integrin engagement results in recruitment and activation of several signalling proteins such as protein kinases FAK (focal adhesion kinase) and Src that play central role in the regulation of cell motility.

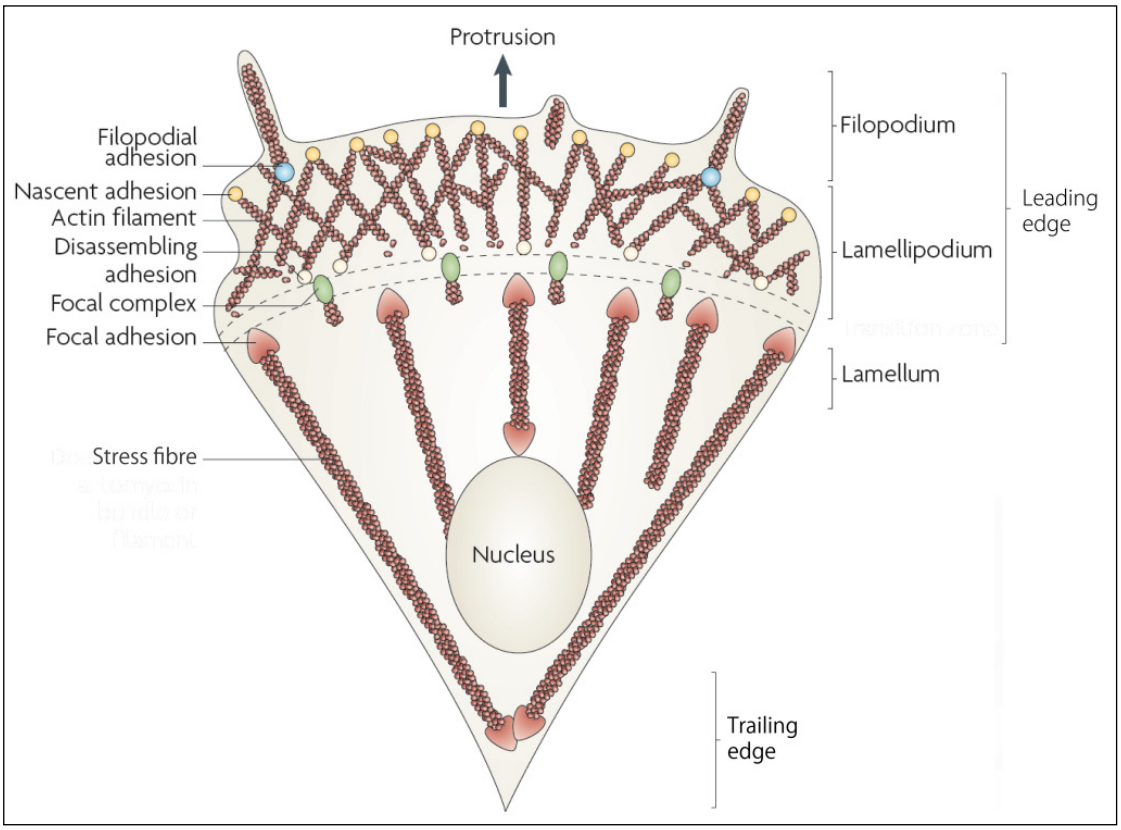

Figure 2.5 – The architecture of cell adhesions and actin cytoskeleton in migrating fibroblast cell. The migrating fibroblast cell exhibits several typical features. In order to migrate, the cell undergoes polarization that leads to formation of leading and trailing edge. Following the polarization the cell forms three types of regions – filopodium, lamellipodium and lamellum, driven by polymerization of actin filaments. Subsequently, nascent adhesions are formed within the lamellipodium. The nascent adhesions either disassemble or undergo maturation at the lamellipodium/lamellum interface. The maturation of the nascent adhesions to focal complexes and focal adhesions is accompanied by the bundling of actin filaments and actomyosin contractility that stabilizes the adhesion and increases the adhesion size. Modified model adapted from (Parsons et al., 2010).

By connecting the actin cytoskeleton to the ECM, focal adhesions serve as a traction sites for generation of force and for forward translocation of the cell body. This propulsion force is mediated by a protein motor myosin II that crosslinks actin filaments

and uses energy from ATP hydrolysis to move antiparallel actin filaments past each other (Vicente-Manzanares et al., 2009). The actomyosin contractility is required not only for the cellular movement, but also for the maturation and disassembly of the cell adhesions. The activity of myosin II is regulated by posttranslational modifications of its regulatory chain and is controlled by several protein kinases and phosphatases including protein kinases ROCK (Rho-associated, coiled-coil containing protein kinase 1) and MLCK (myosin light chain kinase) or MLC phosphatase (Raftopoulou and Hall, 2004).

The last part of the migration cycle is focal adhesion disassembly that allows the cell to dispose of adhesive links at the leading and trailing edge that are not required anymore. At the leading edge, the disassembly occurs at the lamellum-lamellipodium interface and leads to removal of nascent adhesions that are not subject of subsequent maturation. The disassembly of the adhesions located at the rear of the cell literally removes the brakes and enables the cell to move forward (Parsons et al., 2010).

The process of cell migration is precisely controlled by the coordination of multiple signalling pathways. The establishment of cell polarity, actin polymerization, actomyosin contractility and focal adhesion turnover are key cellular events during the cell locomotion. The ERK signalling cascade plays an important role in the regulation of cell motility (Huang et al., 2004). The regulation of actin polymerization, actomyosin contractility and focal adhesion turnover by the ERK pathway is emerged as a significant factor in cell motility, however, the exact molecular mechanisms of the ERK contribution is poorly understood.

2.3.1.2. The ERK-mediated adhesion disassembly

Efficient cell migration involves repeating steps of the focal adhesion assembly and disassembly at the leading edge and at the rear of the cells. The activation of protein kinases FAK and Src were shown to be the key components of the signalling machinery required for proper regulation of the focal adhesion dynamics and cell motility (Ilic et al., 1995; Klinghoffer et al., 1999). FAK and Src exist in a complex that is activated upon the integrin engagement and that regulates activity of many downstream pathways.

Several studies suggested that the ERK signalling is downstream of FAK and Src. The ERK activation by integrins requires the FAK-Src signalling and the active

ERK to localize to focal adhesions in a FAK and Src dependent manner (Fincham et al., 2000; Slack-Davis et al., 2003; Vomastek et al., 2007). Conclusive evidence confirming the involvement of the ERK pathway in the focal adhesion disassembly was brought by the study showing that inhibiting the function of FAK, Src, and protein kinase ERK resulted in significant impairment of the adhesion disassembly (Webb et al., 2004). Moreover, the expression of constitutively active MEK rescued the rate of the focal adhesion disassembly in FAK-null cells. These data suggest that the focal adhesion disassembly is regulated by a linear pathway consisting of FAK, Src and ERK.

The molecular mechanism(s) by which ERK regulates the focal adhesion disassembly is not clear and only few candidate proteins have been identified so far. The regulation of actomyosin contractility, calpain-mediated proteolysis of focal adhesion components and paxillin phosphorylation has been proposed as a mechanism for focal adhesion disassembly.

The ERK-dependent regulation of the focal adhesion disassembly is mediated through activation of protein kinase MLCK because the absence of MLCK resulted in significant decrease in the adhesion disassembly rate (Webb et al., 2004). This notion is supported by the finding that protein kinase ERK localizes to focal adhesions. The localization is mediated by the activity of Src and results in phosphorylation of MLCK (Fincham et al., 2000). MLCK is a Ser/Thr protein kinase that phosphorylates serine residues in MLC which activates myosin II and drives generation of contractile forces required for the reorganization of cytoskeletal structures allowing the adhesion turnover (Totsukawa et al., 2004). In addition to the direct activation of MLCK, the ERK signalling cascade activates MLCK by an alternative pathway comprising of GTPase RhoA and its downstream effector ROCK (Figure 2.6). This pathway controls activity of myosin II and consequently the actomyosin contraction. The GTPase RhoA activates protein kinase ROCK that phosphorylates and thereby inactivates MLC phosphatase which leads to increase of the MLC phosphorylation and actin-myosin II association. Pritchard and colleagues showed that the ERK signalling cascade is linked to the Rho pathway and that the absence of B-Raf results in enhanced motility due to the elevation in ROCK expression and attenuation of Ser3 phosphorylation in protein cofilin (Pritchard et al., 2004). Cofilin is a severing factor that facilitates rapid turnover of actin filaments. The phosphorylation on Ser3 prevents the cofilin-actin association and blocks the actin filament depolymerization (Agnew et al., 1995).

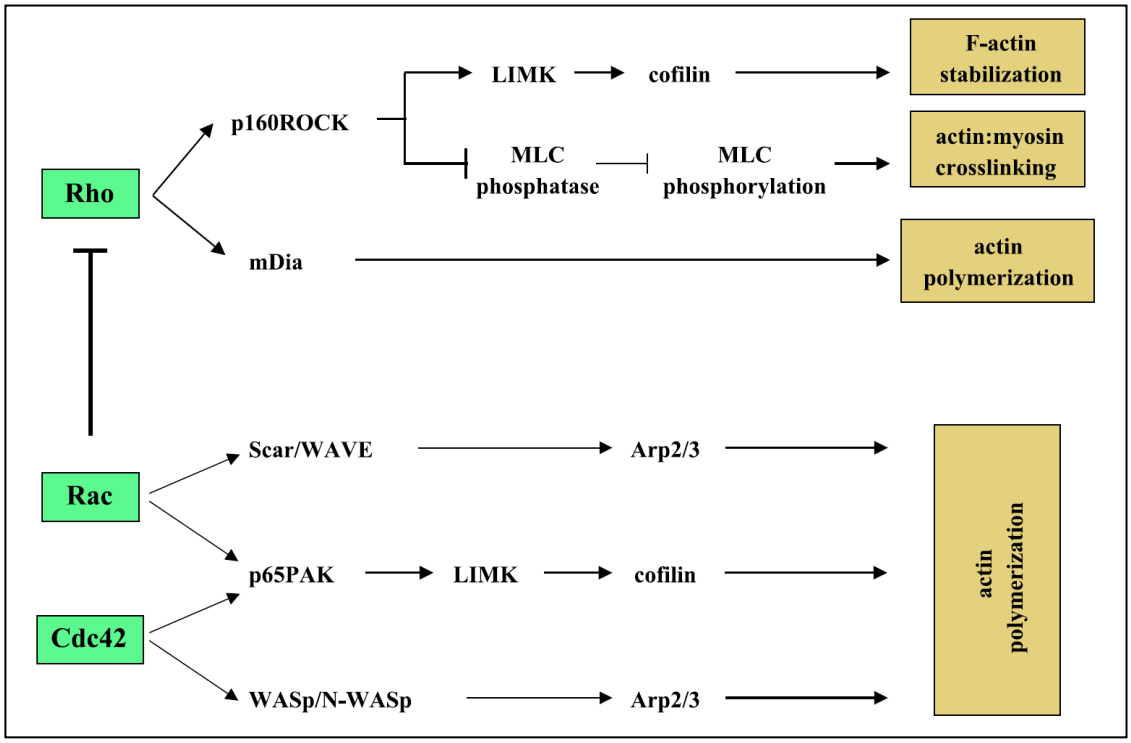

Figure 2.6 – Rho GTPases are key regulators of cell migration. Rho regulates actin cytoskeleton through mDia and ROCK. ROCK phosphorylates LIM kinase and MLC phosphatase, leading to cofilin phosphorylation and increase in MLC phosphorylation, respectively. Rac and Cdc42 both regulate actin polymerization through the WASp/WAVE family of proteins that act through the Arp2/3 complex, and via PAK kinase acting on LIM kinase. Model adapted from (Raftopoulou and Hall, 2004).

Adaptor protein paxillin is another migration-specific substrate of the ERK signalling cascade. Paxillin is one of the integral components of the cell adhesions that interacts with the complex of protein kinases FAK and Src (Deakin and Turner, 2008). The association with the FAK-Src complex is enhanced by the ERK-mediated phosphorylation of paxillin on serine residue 83 and consequently leads to activation of FAK and Rac and to rapid focal adhesion turnover and lamellipodial extension (Ishibe et al., 2004).

Calpain, a cysteine calcium-dependent protease, is another potential ERK target involved in the regulation of focal adhesion disassembly. ERK phosphorylates calpain on serine residue 50, thereby increasing the calpain activity. Active calpain cleaves the components of cell adhesions, FAK and talin, uncouples them from cytoskeletal structures and thus promotes cell migration (Carragher et al., 2003; Franco and Huttenlocher, 2005). Interestingly, calpain-mediated proteolysis requires an assembly of

a complex consisting of calpain, FAK and ERK. The formation of the ternary complex facilitates the translocation of the whole complex to cell periphery. When associated, ERK activates calpain, which leads to calpain-induced cleavage of FAK at distinct sites and subsequent turnover of focal adhesions (Carragher et al., 2003; Sawhney et al., 2006; Chan et al., 2010).

Interestingly, recent study suggested that the ERK signalling cascade regulates cell motility by targeting the Rac pathway. Specifically, ERK phosphorylates WAVE2 and Abi1, two components of WRC (WAVE2 regulatory complex) that is responsible for the activation of actin polymerization factors Arp2/3 (Figure 2.6). Thus, it is tempting to speculate that ERK coordinates the adhesion disassembly with the WRC activation and actin polymerization to promote productive leading edge advancement during cell migration (Mendoza et al., 2011).

Recent study extended the list of the proteins necessary for the ERK-mediated adhesion disassembly by RACK1.

2.3.2. The regulation of cell migration by scaffold protein RACK1

RACK1 is a scaffold protein involved in signal transduction through various intracellular signalling pathways. RACK1 associates with numerous proteins and contributes to many important biological functions. Of particular importance seems to be the ability of RACK1 to link the ERK signalling pathway to mitogenic signals coming from the outside environment and subsequently present the pathway to its downstream effectors. This could be achieved by formation of a multiprotein complex, a signalosome, in which all the key signalling components are bound.

2.3.2.1. History, structure and function of RACK1

RACK1, a Gβ (G protein β subunit) homologue, is a 36-kDa protein and a member of a tryptophan-aspartate repeat (WD-repeat) family. Originally found as a binding partner of PKCβII (protein kinase CβII), RACK1 was named receptor for activated protein kinase C (Ron et al., 1994). Since then, numerous proteins interacting with RACK1 have emerged and approximately 80 RACK1-associated proteins have been reported (Adams et al., 2011). Through such a multitude of binding partners RACK1 contributes to various cellular processes ranging from cell proliferation and

apoptosis to regulation of cell motility. Particularly the RACK1-mediated regulation of cell migration has been shown to be of great importance and will therefore be discussed in following sections.

As mentioned, RACK1 belongs to the WD-repeat containing family of proteins. The WD-repeat proteins are evolutionarily conserved and can be found in prokaryotes and eukaryotes (Li and Roberts, 2001). To date, over 100 members of the WD-repeat family have been identified. The WD-repeats are sequences of typically 44 to 60 amino acids with WD dipeptide at a C terminus. All members of the family contain at least four WD-repeats that form a WD domain. This domain typically adopts β-propeller structure formed by blades arranged around a central axis (Figure 2.7) (Smith, 2008). The majority of the WD-repeat proteins are involved in intracellular signalling because they commonly function as scaffolding proteins. The best studied WD-repeat protein is Gβ of heterotrimeric G proteins, which forms complex with subunit Gγ. The Gβγ reversibly interacts with Gα which results in formation of Gαβγ heterotrimer that associates with GPCR and links extracellular stimuli to downstream signalling pathways (Sondek and Siderovski, 2001).

RACK1 contains seven WD-repeats that adopt the characteristic β-propeller structure (Figure 2.7). All protein interactions, direct or via large multiprotein complexes, are mediated by the blades of the β-propeller fold. This way RACK1 interacts with SH2, PH or C2 domains located in RACK1 binding proteins (Adams et al., 2011).

RACK1 is encoded by *GNB2L1* gene, which is strongly conserved throughout the evolution as documented by the 43-76% sequence identity when comparing RACK1 from *Tetrahymena thermophila, Saccharomyces cerevisiae, Arabidopsis thaliana* and *Drosophila melanogaster* (Adams et al., 2011). RACK1 is highly expressed in most tissues (Chou et al., 1999) and imbalance in RACK1 expression can lead to serious defects. For example, the deregulated RACK1 expression contributes to brain pathologies (Peyrl et al., 2002) or is associated with cancer (Al-Reefy and Mokbel, 2010; Cao et al., 2010). This underlines the importance of RACK1 and the requirement for tight regulation of its expression.

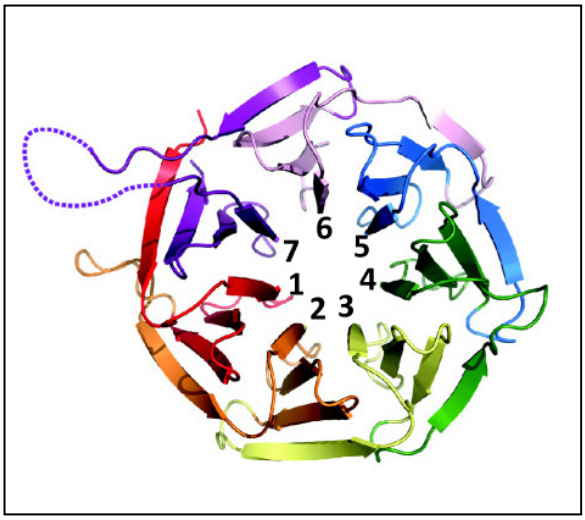

Figure 2.7 – Structure of RACK1 scaffold protein from *Arabidopsis thaliana*. The structural model illustrates typical β-propeller structure of RACK1 WD domain. The β-propeller structure is formed by seven blades (WD repeats) that mediate interaction with distinct targets of RACK1. Model adapted from (Adams et al., 2011).

2.3.2.2. RACK1 in the regulation of cell migration

First suggestion that RACK1 might contribute to the regulation of cell migration came from studies showing that RACK1 binds to β integrin subunits (Liliental and Chang, 1998). Direct evidence proving the involvement of RACK1 in the control of cell motility was brought by studies showing that cells lacking RACK1 are less motile (Cox et al., 2003; Doan and Huttenlocher, 2007; Vomastek et al., 2007). It was found that RACK1 also associates with downstream effectors of integrins and protein kinases FAK and Src (Chang et al., 1998; Serrels et al., 2010). The regulation of the FAK-Src functions seems to represent a major mechanism by which RACK1 regulates cell migration. However, the exact mechanism by which RACK1 controls cell motility remains poorly understood.

2.3.2.2.1. Regulation of cell migration by RACK1-FAK-Src signalling

One of the most important functions of RACK1 during cell migration is to read signals coming from the integrin-FAK-Src signalling and then to transmit these signals to downstream effectors. These effectors then regulate cell motility at several levels.

Reorganization of actin cytoskeleton and focal adhesion disassembly are the most prominent steps.

Probably the best understood function of RACK1 in the cell migration is the regulation of focal adhesion disassembly (Doan and Huttenlocher, 2007; Vomastek et al., 2007). The focal adhesion disassembly is regulated by a linearly organized signalling pathway consisting of integrins-FAK-Src-Raf/MEK/ERK proteins (Webb et al., 2004). In addition, active protein kinase ERK localizes to focal adhesions where it presumably induces the focal adhesion disassembly by phosphorylating key focal adhesion proteins (Fincham et al., 2000; Vomastek et al., 2007). It was shown that RACK1 functions as a scaffold protein of the ERK pathway that binds all three core protein kinases Raf, MEK and ERK and facilitates their activation in response to cell adhesion. Attenuation of RACK1 expression led to reduction of active ERK in focal adhesions, increased focal adhesion length and decreased rate of adhesion disassembly. As a consequence, cells displayed decreased motility toward the extracellular matrix protein fibronectin (Vomastek et al., 2007). It was suggested that RACK1 enhances the activation of the ERK signalling module by connecting it with the upstream activators, the FAK-Src complex and integrins, and downstream effectors localized in focal adhesions and that these effector proteins are responsible for the adhesion disassembly (Vomastek et al., 2007). Below (Figure 2.8) is a schematic representation of how RACK1 links the ERK pathway to the upstream and downstream targets.

There are at least two other mechanisms that relate the RACK1, FAK and Src signalling to the regulation of cell migration. RACK1 regulates Src-dependent phosphorylation and activation of p190RhoGAP (Miller et al., 2004). p190RhoGAP is a GTPase-activating protein involved in inactivation of RhoA in response to integrin-mediated adhesion that contributes to actin cytoskeleton remodelling during cell spreading, membrane protrusion and cell polarization (Arthur and Burridge, 2001).

Recent reports suggest that RACK1 is involved in a novel pathway that mediates directional movement in squamous cancer cells. This pathway links FAK, RACK1 and PDE4D5 to integrin signalling in newly forming adhesions to promote cell polarity (Serrels et al., 2011; Serrels et al., 2010).

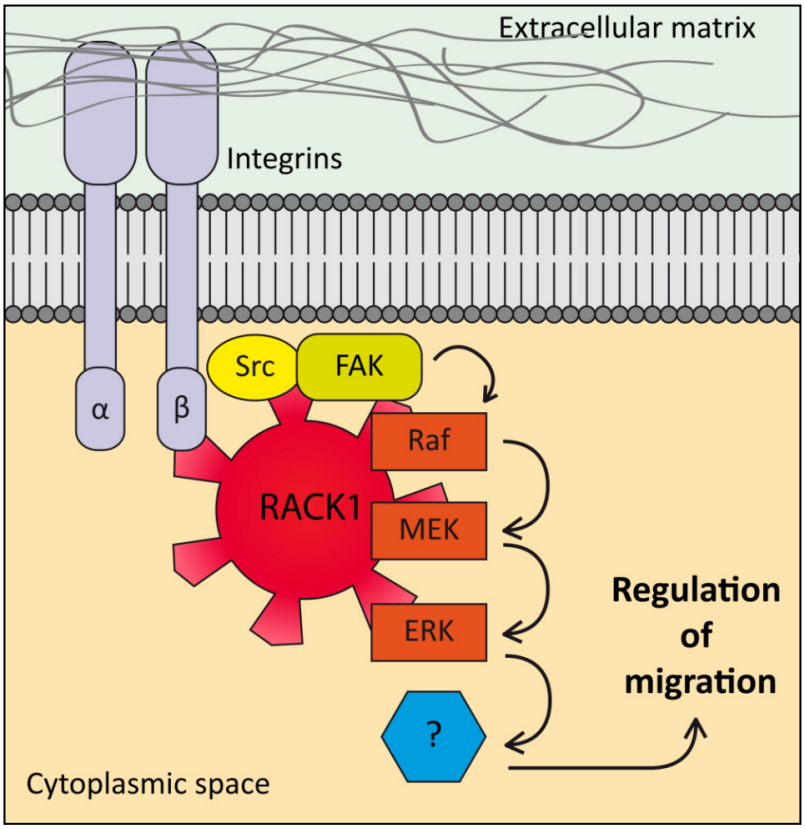

Figure 2.8 – Model of RACK1-ERK signalling pathway. RACK1 links signals from integrins and FAK-Src complexes to the components of the ERK cascade and facilitates its activation upon cell adhesion. By binding all relevant components RACK1 connects the ERK signalling module with upstream activators (integrins, FAK-Src complex) and facilitates the signal transduction towards downstream targets. The downstream targets that are involved in the regulation of cell motility remain uncharacterized.

In addition to regulation of the activity of signalling proteins downstream of FAK and Src, RACK1 has the ability to directly regulate the FAK/Src activity. Src phosphorylates RACK1 on several tyrosine residues and creates a binding site for the Src SH2 domain (Chang et al., 1998). When associated with Src, RACK1 inhibits the activity of Src which consequently leads to decreased level of tyrosine phosphorylation of Src targets during cell adhesion and motility (Mamidipudi et al., 2004; Miller et al., 2004). The release of RACK1 from Src is mediated by IGF-1 signalling (Kiely et al., 2005). RACK1 can as well associate with and modulate activity of the protein kinase FAK. The RACK1-FAK interaction is required for efficient activation of FAK by autophosphorylation on Tyr397 in response to adhesion signals (Kiely et al., 2009).

Suppression of RACK1 expression disrupts the FAK activity and leads to defective cell adhesion and cell spreading (Kiely et al., 2005).

2.3.2.2.2. Regulation of cell migration by RACK1-Akt and RACK1-PKC signalling

RACK1 also regulates cell migration by controlling additional signalling pathways including PKC or Akt. The Akt pathway contributes to the regulation of cell motility through reorganization of actin cytoskeleton. Akt is negatively regulated by protein phosphatase 2A (PP2A) that interacts with RACK1. PP2A competes for the binding of RACK1 with β1 integrin. When the cell is stimulated by cell adhesion, PP2A is rapidly displaced from RACK1 allowing the activation of the Akt pathway and cell migration (Kiely et al., 2006).

In addition to the Akt pathway, RACK1 mediates activation of PKCε by linking it with integrin β chains. Thus, RACK1 contributes to PKCε-induced cell adhesion and migration (Besson et al., 2002). However, the molecular mechanism is not understood.

Although the evidence for positive modulation of migration by RACK1 is quite abundant, it is necessary to mention that few studies describe RACK1 as a negative migration regulator. For example, Buensuceso and colleagues observed increased cell motility and higher number of actin stress fibres and focal contacts in CHO cells stably overexpressing RACK1 (Buensuceso et al., 2001). Similarly, it was discovered that RACK1 negatively regulates directional cell migration by binding and inhibiting Gβγ. This resulted in repression of PLCβ and PI3Kγ pathways because RACK1 competes with both proteins for the binding of Gβγ (Chen et al., 2008). Without a doubt further studies are necessary to fully understand the RACK1 function as a scaffold as well as how RACK1 regulates the process of cell migration.

2.3.2.3. RACK1-mediated regulation of protein translation

Much of the knowledge we currently have about the function of RACK1 comes from studies describing RACK1 as a regulator of protein translation. RACK1 binds to 40S ribosomal subunit (Sengupta et al., 2004) and recruits multitude of signalling proteins, most importantly PKCβII. When present at the ribosome, PKCβII

phosphorylates eukaryotic initiation factor 6 bound to 60S subunit and triggers its release which leads to assembly of the ribosome (Ceci et al., 2003). In contrary to these findings, some studies suggest that ribosomal fraction of RACK1 may repress gene expression by recruiting components of microRNA-induced gene silencing complex (Jannot et al., 2011) or by inducing a nascent peptide-dependent translation arrest (Kuroha et al., 2010). Thus, it is likely that the contribution of RACK1 to translation depends on the proteins that are recruited onto the ribosomes and that the set of the protein regulators available at the given moment is cell type-specific.

There is ample evidence that localized mRNA translation at the leading edge of migrating cells is necessary for efficient cell migration (Mili and Macara, 2009). Considering the fact that RACK1 regulates both protein translation and cell motility, it is tempting to speculate that RACK1 contributes to cell migration also by promoting the location-specific protein synthesis.

2.4. The aim of diploma thesis

Our current knowledge about the protein RACK1 suggests the existence of complex mechanism by which RACK1 regulates cell motility. RACK1 serves as a sensor for various extracellular signals and activates several pathways that act in parallel. The ERK signalling cascade is one of these pathways the activation of which promotes cell migration. The guiding hypothesis underlying this work is that RACK1 as a scaffold for the ERK pathway helps to transmit the signal towards migration-specific substrates. If we take into account all available information concerning the ERK-mediated regulation of cell migration and the involvement of the scaffold protein RACK1 in this process, it is evident that the number of the discovered migration-specific ERK targets is far from being final. Therefore, the aim of this study is to identify new potential effector proteins utilized by the ERK signalling cascade and RACK1 protein during regulation of cell motility.

3. Materials and methods

3.1. Materials

3.1.1. Cell lines and cell culture

RAT-2 and COS-1 cell lines were maintained in DMEM (Dulbecco's modified Eagle's medium; Gibco) supplemented with 10% FBS (fetal bovine serum; Gibco).

3.1.2. List of primary antibodies for western blot

Name	Dilution	Source of origin	MW*	Manufacturer
Acetylated tubulin	1:2 000	mouse monoclonal	50 kDa	Sigma
p-Cofilin (S3)	1:1 000	rabbit monoclonal	18 kDa	Cell Signalling
ERK2	1:5 000	mouse monoclonal	42 kDa	provided by M.J. Weber
p-ERK2 (T-E-Y motif)	1:1 000	rabbit polyclonal	42 kDa	provided by M.J. Weber
FLAG	1:1 000	rabbit polyclonal		GenScript
HA (12CA5)	1:1 000	mouse monoclonal		provided by M.J. Weber
Glutamylated tubulin	1:500	rabbit polyclonal	50 kDa	Millipore
p-Paxillin (S83)	1:500	rabbit polyclonal	68 kDa	ECM Biosciences
RACK1	1:1 000	mouse monoclonal	36 kDa	Santa Cruz
RSK	1:1 000	mouse monoclonal	90 kDa	BD Transduction Laboratories
p-RSK (T359)	1:1 000	rabbit polyclonal	90 kDa	GenScript
p-RSK (T359/S363)	1:1 000	rabbit polyclonal	90 kDa	Cell Signalling
p-RSK (T573)	1:1 000	rabbit polyclonal	90 kDa	Cell Signalling

* MW = molecular weight

3.1.3. List of secondary antibodies for western blot

Name	Dilution	Manufacturer
Goat anti-mouse IgG HRP-conjugated antibody	1:10 000	Sigma
Goat anti-rabbit IgG HRP-conjugated antibody	1:5 000	Sigma
Goat anti-rabbit IgG HRP-conjugated antibody	1:2 000	Cell Signalling

3.1.4. List of primary antibodies for immunofluorescence microscopy

Name	Dilution	Source of origin	Manufacturer
F-actin (phalloidin conjugated with Texas Red-X)	1:50		Invitrogen
Paxillin	1:100	mouse	Cell Signalling
Vinculin	1:300	mouse	Sigma

3.1.5. List of secondary antibodies for immunofluorescence microscopy

Name	Colour	Dilution	Manufacturer
Alexa fluor 488 anti-mouse	green	1:200	Invitrogen
Alexa fluor 488 anti-rabbit	red	1:200	Invitrogen

3.1.6. List of inhibitors

Name	Function	Concentration	Manufacturer
BI-D1870	inhibitor of RSK1/2/3	10 μM	Axon Medchem
C3 transferase	inhibitor of Rho GTPases	1 μg/ml	Cytoskeleton
U0126	inhibitor of MEK1/2	10 μM	Promega
Y-27632	inhibitor of ROCK1	20 μM	Calbiochem

3.1.7. List of siRNAs

Name	Target sequence	Manufacturer
siRNA “1”	AAGGTGTGGAATCTGGCTAAC	Qiagen
siRNA “2”	GCTAAAGACCAACCACATTTT	Qiagen
siRNA “4”	AACTGTCCAGGATGAGAGT	Eurofins MWG Operon

3.1.8. List of plasmid constructs

Name of construct	Cloned in
FLAG-RACK1 (Vomastek et al., 2007)	pcDNA3 (Invitrogen)
HA-RSK	pKH3 (Addgene)

3.2. Methods

3.2.1. Transient transfections

3.2.1.1. Transfection by calcium phosphate co-precipitation

Transfection by calcium phosphate co-precipitation followed standard protocol described in (Graham and van der Eb, 1973) and (Wigler et al., 1978). One day prior to transfection, cells were seeded on 60 mm dishes or 6-well plate to reach 50-60 % of confluency at the time of the transfection. On the day of the transfection, two sets of 15ml tubes were prepared. Into one tube 2x HEBS (HEPES buffered saline; pH 7-7.2) was added and the second tube was supplemented with mixture of siRNA and 1x TE (Tris-EDTA) with 2.5M $CaCl_2$ in 10mM HEPES (pH 7.2). Mock-transfection control cells received transfection reagent only. Then the content of the second tube was added dropwise to the first tube to start the co-precipitation process. The mixture was incubated for 30 min at room temperature and added to cells afterwards. After 5 hours, the transfection medium was removed and cells were analyzed 24 to 48 hours posttransfection.

3.2.1.2. PolyFect transfection

PolyFect transfection (Qiagen) method was employed when transfecting cells with plasmid constructs for co-immunoprecipitation experiments. The PolyFect transfection followed manufacturer's protocol optimized for transient transfection of NIH 3T3 cells in 6-well plate, 60mm and 100mm dish. After 24 to 48 posttransfection, cell lysates were processed (see section 3.2.3.).

3.2.2. siRNA-mediated gene silencing

To silence the RACK1 gene, double-stranded small interfering RNAs (siRNAs) were synthesized. The siRNAs were transfected into RAT-2 cells by calcium phosphate co-precipitation and 24 to 72 hours posttransfection cells were processed as described in the following section.

3.2.3. Sample preparation and SDS-PAGE

To prepare lysates for SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 1 mM EDTA). Cells were lysed in FLAG lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM EDTA, 0.5 mM EGTA, pH 7.3) supplemented with phosphatase inhibitor mix I (Serva) and protease inhibitor mix G (Serva) when preparing samples for protein co-immunoprecipitation experiments.

SDS-PAGE and western blot followed standard protocols. Cell lysates were boiled in SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue) for 10 minutes at 100 °C. Protein concentration of samples was measured by BCA protein assay kit (Pierce). Samples were loaded into polyacrylamide gel and run in SDS running buffer (25mM Tris, 192mM glycin, 0.1% SDS).

3.2.4. Western blot and immunodetection

By SDS-PAGE resolved proteins were transferred from the gel onto nitrocellulose membrane (Sigma) by blotting in transfer buffer (48mM Tris, 39mM Glycin, 0.0375% SDS, 20% methanol). The membranes were stained with 0.2% Ponceau S to locate transferred proteins and destained in distilled water afterwards. Subsequently, the membranes were blocked for 1 hour in blocking solution (5% BSA [bovine serum albumin] in PBS-T [PBS supplemented with 0.05% Tween-20], supplemented with 0.01% NaN_3) at room temperature and then incubated with primary antibodies overnight at 4 °C. Primary antibodies were diluted in antibody diluent solution (5% BSA in PBS-T, supplemented with 0.01% NaN_3). Afterwards, the

membranes were washed three times in PBS-T and incubated for 1 hour at room temperature with secondary antibodies conjugated with HRP (horse radish peroxidase) diluted in PBS-T. After the incubation with the secondary antibodies, the membranes were washed three times in PBS-T and incubated with SuperSignal West Pico Chemiluminiscent Substrate (Pierce) for 5 minutes. The chemiluminiscent signal was detected by autoradiography films and developed by automatic Agfa Curix 60 film processor.

When re-probing the membranes, the membranes were washed with hot stripping buffer (62.5 mM Tris HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 20 minutes and re-blocked with blocking solution prior to next primary antibody incubation.

3.2.5. Protein co-immunoprecipitation

COS-1 cells were cultured in 100mm dishes. When 40-60 % confluent, cells were transiently transfected with FLAG-RACK1 and HA-RSK plasmid constructs following the PolyFect transfection protocol. Cells were harvested 24 to 48 hours posttransfection in FLAG lysis buffer supplemented with phosphatase and protease inhibitors (see section 3.2.3.). Pre-cleared cell extracts were incubated for 2 hours with M2 anti-FLAG affinity resin (Sigma) at 4°C to form immunocomplexes. The affinity resin was then washed three times with FLAG lysis buffer, boiled with SDS loading buffer for 10 minutes at 100 °C, resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Sigma).

Immunoblot analysis was carried out with anti-FLAG (GenScript) and anti-HA 12CA5 antibodies. Washes, incubation with secondary antibodies and chemiluminiscence detection were performed as described above (see section 3.2.4.).

3.2.6. Cell stimulation

To determine activation status of analysed proteins in response to EGF (Upstate), cells were serum deprived for 4 hours or overnight and then stimulated with EGF (10 ng/ml) for 30 minutes. Cells were lysed in RIPA buffer and samples were processed (see section 3.2.3.).

3.2.7. G-LISA RhoA activation assay

G-LISA RhoA activation assay (Cytoskeleton) is a technique that allows rapid measurement of Rho activation from cell and tissue samples and it can be used as an alternative to conventional pull-down assay. The G-LISA assay is based on a Rho-GTP-binding protein linked to the wells of 96-well plate. Active GTP-bound Rho binds to the wells while inactive GDP-bound Rho is removed during washing. The bound active RhoA is detected with a RhoA specific antibody and secondary antibody coupled to HRP. The level of RhoA activity is assessed by colorimetric reaction and by comparing signals from activated and non-activated cell lysates.

Manufacturer's instructions were followed to measure RhoA activity in cell lysates from mock-transfection control and RACK1 knockdown cells.

3.2.8. Fluorescence microscopy

All fluorescent images were acquired using Olympus Cell-R imaging station (Olympus IX81 inverted microscope, MT20 illumination system and Olympus FV2T CCD camera).

To prepare samples for widefield fluorescent imaging, cells were seeded on glass coverslips coated with fibronectin (1.0 μg/ml, Millipore). Cells were washed two times with PBS, fixed in 1% paraformaldehyde in PBS for 20 minutes and permeabilized with 0.5% Triton in PBS for 5 minutes. The cells were then washed twice with PBS and blocked in 20% normal goat serum (PAA) in PBS for 1 hour. Subsequently, the cells were incubated with primary and secondary antibodies (diluted in PBS supplemented with 5% normal goat serum) for 1 hour each as listed above. Vectashield mounting medium with DAPI (Vector Laboratories) was used for mounting the glass coverslips.

3.2.9. Analysis of cellular protrusivity

Prior to the analysis of cellular protrusivity, cells were transiently transfected with siRNA silencing the RACK1 gene using the standard calcium phosphate co-precipitation protocol. After 48 hours, phase contrast images were acquired every 10 seconds for 5 minutes. Cellular protrusivity was analysed by creating kymographs of

cell periphery. The kymographs are graphical representations of a spatial position detected over time in a stack of images (each pixel on x axis represents one captured image) and they were constructed using ImageJ software and Kymograph plug-in (http://www.embl.de/eamnet/html/body_kymograph.html).

3.2.10. Calcein AM staining

Calcein AM staining was carried out to semi-automatically analyze the shape of RAT-2 cells. Calcein AM (Calbiochem) is a cell-permeant and non-fluorescent compound that is widely used for determining cell viability. In live cells, the AM group is hydrolyzed by intracellular esterases and highly fluorescent calcein molecule is formed. Calcein is retained in cytoplasm and could therefore be used to visualize whole cells.

To perform the staining, cells were incubated with 0.5 μM calcein AM for 45 minutes. The culture medium was then replaced with fresh medium and fluorescence images were acquired using Olympus Cell-R microscope. Cell shape was analyzed using ImageJ software as described in the following section.

3.2.11. Roundness index determination

Using the ImageJ software, all fluorescent images were processed (binarized and thresholded) to acquire cell outlines. These images were thereafter analyzed to obtain Feret's diameter (the length from tip-to-tail of the cell) and cell area to calculate roundness index using the following equations:

1) $\pi \times (\text{Feret's diameter}/2)^2 = \text{area of ideal circle}$

(area occupied by the cell provided it has perfect circular shape)

2) **Roundness index (RI) = measured area/ideal area**

(value determining the cell circularity on the scale from 0 to 1, with 1 representing the perfect circular shape)

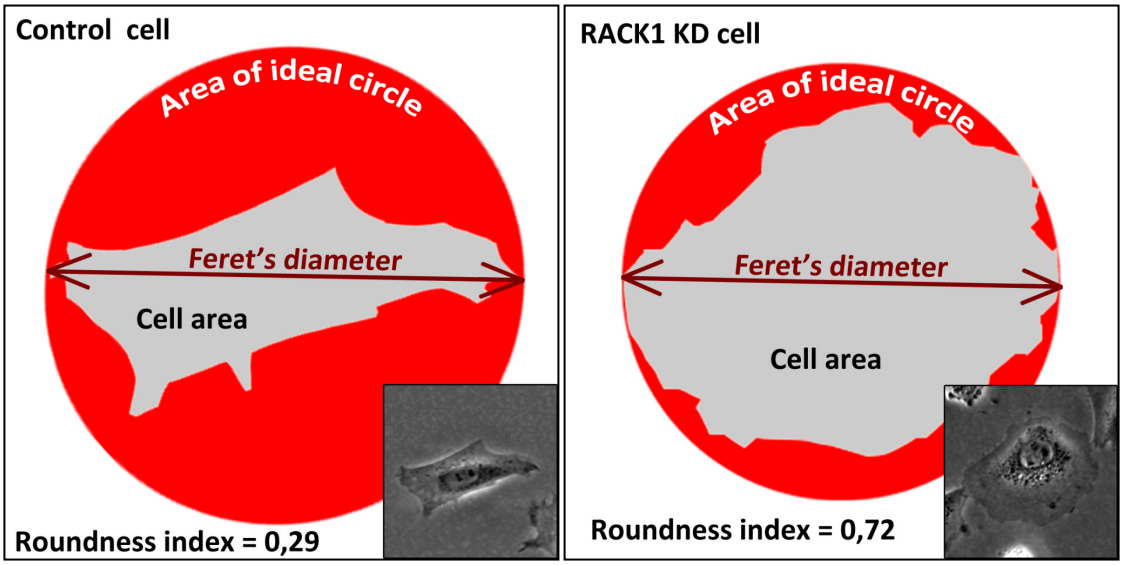

Figure 3.1 - Representation of roundness index measurement. Cells that adopt conical shape have significantly lower roundness index than cells with circular shape.

3.2.12. Cell migration assays

3.2.12.1. Wound healing assay

The wound healing assay is a simple method to study directional cell migration *in vitro*. This method mimics cell migration during wound healing *in vivo*. It is based on creating a "wound" in a cell monolayer, capturing the images at regular intervals during cell migration and comparing the images to quantify the migration rate of the cells (Rodriguez et al., 2005).

To carry out the wound healing assay, cells were seeded on 6-well plate coated with fibronectin (1.0 µg/ml, Millipore) to form a monolayer. On the day of the experiment, the wound was made and 4 hours later cell images were captured in regular intervals of 10 minutes for 10 hours. Phase contrast images were acquired using Olympus Cell-R microscope and analysed in ImageJ software. When analysing effects of inhibitors on cellular motility, cells were treated with indicated inhibitors (see section 3.1.6.), which were applied 30 minutes before the end of the 4-hour period.

3.2.12.2. Random cell migration

Tracking the movement of sparsely seeded cells represents another approach to study cellular motility. Unlike the wound healing assay, cells in random cell migration assay do not display uniform movement, but migrate in all directions.

On the day of the experiment, approximately 4 x 10^4 to 8 x 10^4 cells were seeded to fibronectin-coated wells (1.0 µg/ml, Millipore) in 6-well plate. After 2 hours, images were acquired every 10 minutes for 10 hours. When analysing effects of inhibitors on cellular motility, cells were treated as described above (see section 3.1.6.). The inhibitors were applied 30 minutes before the end of the 2-hour period.

All images were acquired using Olympus Cell-R microscope and analysed using ImageJ software. To analyse the movement of individual cells, ImageJ Manual Tracking plug-in was used (http://rsb.info.nih.gov/ij/plugins/track/track.html). To construct plot-graphs and to measure accumulated distance travelled by individual cell over the time and cell directionality, Chemotaxis and Migration Tool 2.0 (Ibidi) was used. The directionality represents ratio of the direct distance from start to end point divided by the total track distance.

Chemotaxis and Migration Tool 2.0:
http://www.ibidi.com/products/sw_img_analysis/p_cm_tool.html

3.2.13. Statistical analysis

Statistical analysis was carried out in GraphPad Prism 3 software. Error bars represent the mean +/- standard deviation. * corresponds with $p < 0.05$, ** with $p < 0.01$ and *** with $p < 0.001$ in a Mann-Whitney non-paired t-test in comparison to control sample. ns = statistically not significant result

4. Results

4.1. RACK1 as a signalosome

We hypothesise that the scaffold protein RACK1 by the ability to interact with numerous proteins enables the formation of a large multiprotein complex, a signalosome. Thereby RACK1 contributes to regulation of various cellular processes. Via the signalosome RACK1 regulates several parallel pathways and at the same time facilitates their spatial and temporal coordination. The pathways involved in the putative signalosome include PKC, PKA, FAK, Src, Akt and ERK. To understand the function of such a protein, it is important to characterize the cellular processes regulated by RACK1 and to link them to individual signalling pathways. In this section we focused on characterization of phenotype of RACK1 knockdown cells (hereafter referred to as RACK1 KD).

4.1.1. Knockdown of RACK1 induces reorganization of actin cytoskeleton and focal adhesions

To explore the RACK1-dependent functions and to gain some insight into the underlying signalling network, we silenced RACK1 expression by siRNA and observed phenotype of RAT-2 fibroblasts. Upon the knockdown (KD) of RACK1, RAT-2 cells exhibited circular shape (rather than conical that is typical for migrating fibroblasts), formed visibly elongated focal adhesions and displayed reorganized actin cytoskeletal network (Figure 4.1). These results suggested involvement of the ERK signalling pathway as they were in acceptance with previously reported findings that the depletion of RACK1 leads to the elongation of the focal adhesions due to absence of active form of the protein kinase ERK in focal adhesions. The RACK1-mediated relocalization of the active ERK is required for subsequent turnover of focal adhesions and for efficient regulation of cell migration (Vomastek et al., 2007).

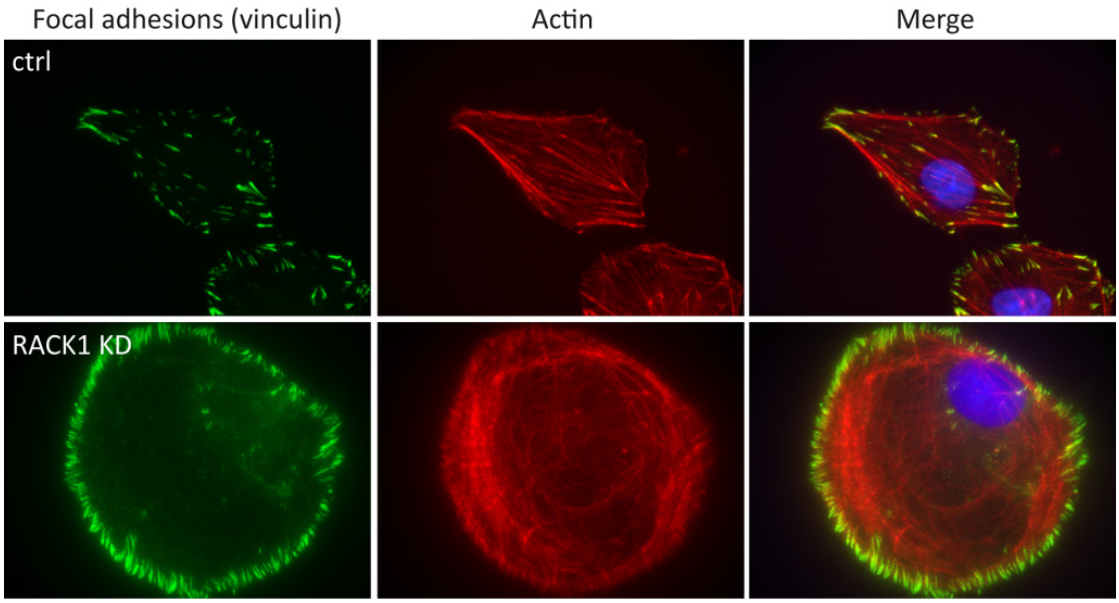

Figure 4.1 – Suppression of RACK1 by siRNA leads to reorganization of actin cytoskeleton and elongation of focal adhesions. RAT-2 cells were transfected with siRNA targeting RACK1 mRNA for 48 hours and plated on glass coverslips coated with fibronectin (1.0 µg/ml). Cells were stained with antibody against vinculin and with phalloidin Texas Red-X to visualize actin filaments. Nuclei were stained with DAPI.

4.1.2. Silencing of RACK1 leads to changes in cellular shape of RAT-2 fibroblasts

Because a shift in a cellular shape is a characteristic feature of the RACK1 KD cells, we sought to quantify this change. To semi-automatically analyze the cellular shape we stained whole cells with Calcein AM (Figure 4.2A). Fluorescent images were analyzed using ImageJ software to determine a roundness index of individual cells. The roundness index is a value that determines on the scale from 0 to 1 how circular (or round) the cell is (with 1 representing the perfect circular shape). The measurement of the roundness index of both control and RACK1 KD cells revealed that the decline in the RACK1 expression leads to significant increase in the roundness index (Figure 4.2B). These data corresponded with our initial observations. Moreover, we measured area of individual cells and found that the RACK1 KD cells cover significantly larger area (Figure 4.2C). Taken together these data indicate that RACK1 is involved in regulation of cellular shape.

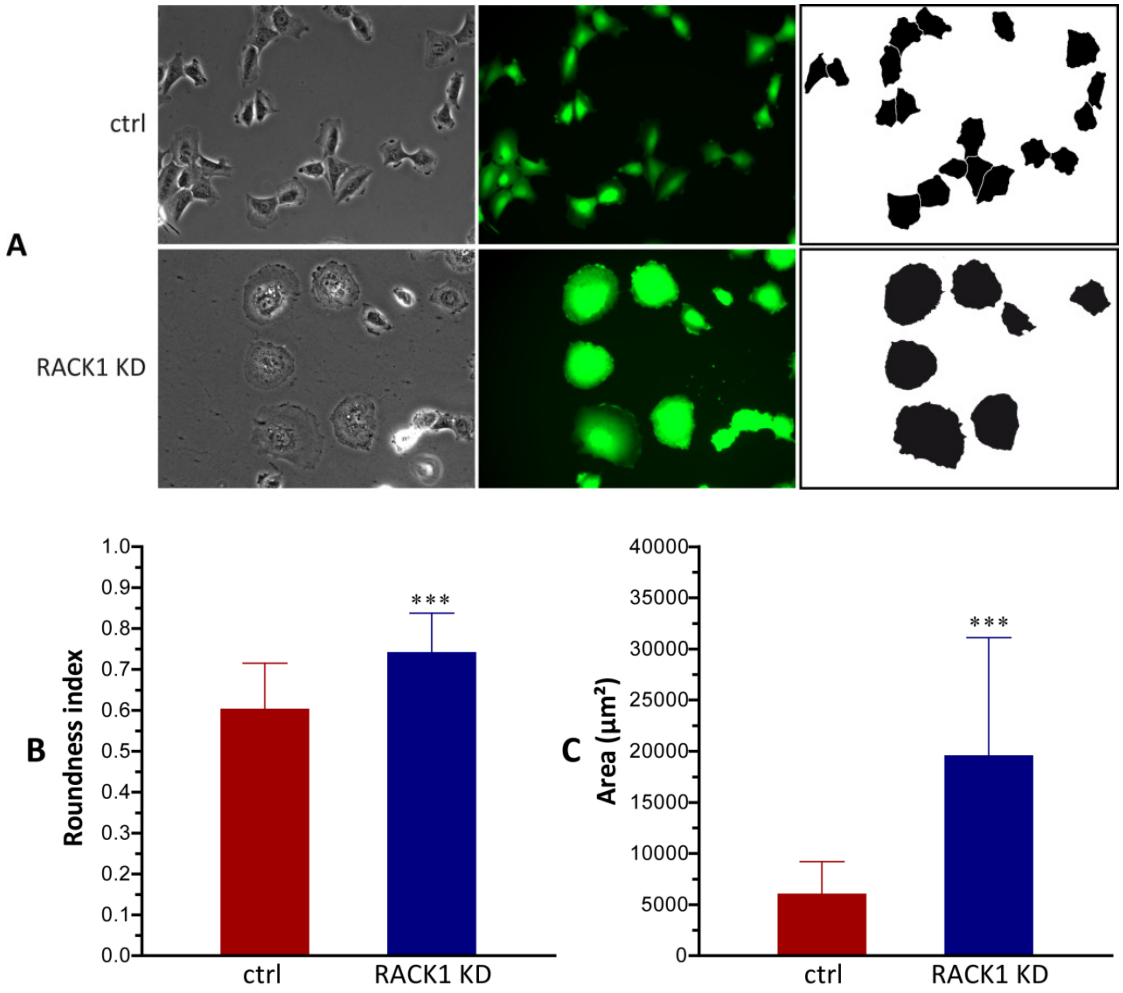

Figure 4.2 – Depletion of RACK1 results in more circular shape and enlarged area occupied by RAT-2 cells. (A) The methodology for cell shape quantification. RAT-2 cells were transfected with siRNA for 48 hours and then stained with Calcein AM for 45 minutes. Captured images were subsequently binarized and analysed using ImageJ. (B) Quantification of roundness index revealed that RACK1-suppressed cells have more circular shape (0.75) than control cells (0.60). (C) Quantification of cell area revealed that RACK1 KD cells occupied significantly larger area than control cells. The images and graphs represent average results from at least three separate experiments.

4.1.3. RACK1 regulates cell protrusivity

Our next aim was to examine, whether the observed defect in actin remodelling in the RACK1 KD cells has any effect on efficiency of lamellipodial extension and formation of membrane protrusions. To assess cell protrusivity, cells were imaged over short period of time by phase contrast microscopy and the cell boundaries were

analyzed by kymographs (Figure 4.3B). The kymographs are graphical representations of a spatial position detected over defined period of time in a stack of images. Each pixel on x axis represents one captured image. We found that periphery of RAT-2 control cells can be divided according to its ability to form membrane protrusions. We determined two types of regions in the control cells – segments actively protruding and quiescent segments producing only limited number of protrusions. Silencing of RACK1 resulted in loss of this apparent segmentation (Figure 4.3A and B).

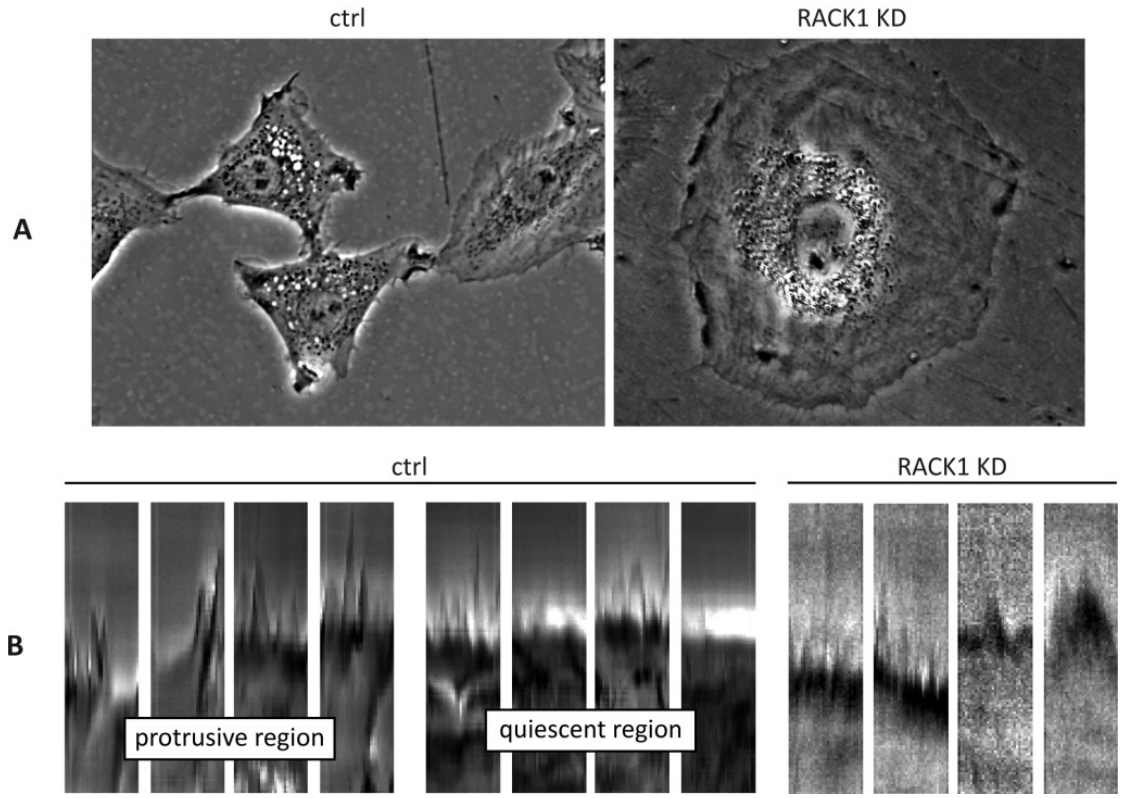

Figure 4.3 – RACK1 is required for regulation of cell protrusivity. (A) RAT-2 cells were transfected with siRNA for 48 hours and plated on dishes with glass bottom coated with fibronectin (1.0 μg/ml). Phase contrast images were subsequently captured every 5 seconds for 5 minutes. (B) Kymograph analysis of cell protrusivity revealed that control cells were segmented into two different regions of plasma membrane – quiescent regions (producing only a limited number of membrane extensions) and protrusive regions (rapidly forming high number of protrusions). RACK1 KD cells completely lacked this segmentation.

Quantification of membrane protrusion length revealed that while the actively protruding regions in the control cells produced long membrane extensions, the quiescent regions and whole plasma membrane of the RACK1 KD cells formed shorter

protrusions (Figure 4.4). These results suggest that RACK1 contributes to the regulation of cell protrusivity.

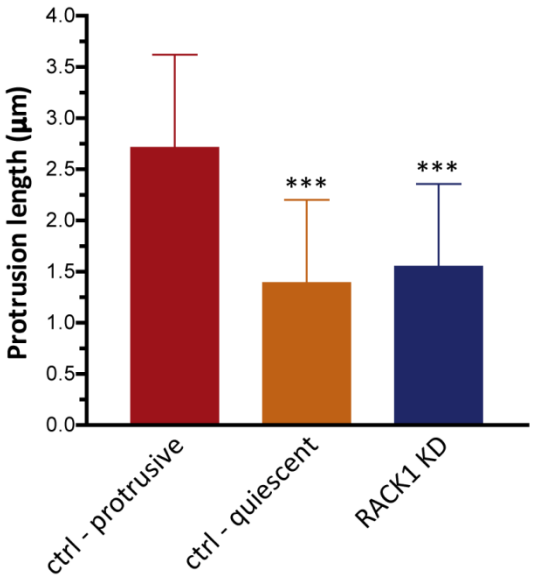

Figure 4.4 - Quantification of cell protrusion length showed that RACK1 KD cells and quiescent regions of control cells form significantly shorter protrusions (approximately 1.5 µm and 1.4 µm, respectively) than protrusive regions (2.6 µm) of control cells. The graph represents results obtained by a single experiment.

At this moment we decided not to continue in the analysis of the cell shape and cell protrusivity for the following reasons: (i) we encountered big technical limitations in some assays such as the determination of cell protrusivity; (ii) the investigation of RACK1 function in the regulation of cell shape became a part of another project solved in the laboratory; and most importantly (iii) the regulation of cell shape and protrusivity depends on other signalling factors functioning in parallel to the ERK pathway. Thus, the further work was focused on the RACK1- ERK dependent processes.

4.2. RACK1-ERK-dependent functions

The organization of the focal adhesion architecture in the RACK1 KD cells, namely the focal adhesion length, strongly resembles the phenotype induced by the inhibition of the ERK pathway. Taken together with numerous published results that the ERK pathway regulates the focal adhesion disassembly and consequently cell motility, our data indicated that the RACK1-mediated signal transduction might involve the

mechanism that directs the ERK signalling cascade towards the regulation of focal adhesion dynamics. In this section we therefore focused on characterization of the mechanism by which RACK1 regulates focal adhesions. In particular, we were interested in identifying effector proteins that are downstream of ERK and RACK1.

4.2.1. RACK1 regulates adhesion-induced, but not serum-induced activation of ERK

It has been shown that RACK1 has a regulatory function in the ERK activation during cell adhesion, but is dispensable when cells are stimulated by growth factors (Vomastek et al., 2007). To investigate the role of RACK1 in the ERK activation, we silenced RACK1 using three siRNAs targeting distinct regions of RACK1 mRNA. The depletion of RACK1 did not affect the activity of protein kinase ERK in RAT-2 fibroblasts cultured in medium supplemented with fetal bovine serum. These data suggest that RACK1 is dispensable for the process of the activation of ERK in cells growing in the presence of serum (Figure 4.5).

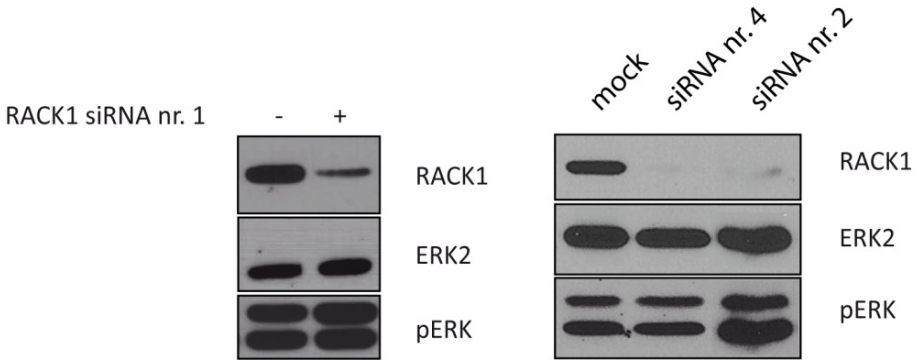

Figure 4.5 – RACK1 silencing does not affect global ERK activity. RAT-2 cells were transfected with three different siRNAs targeting distinct regions of RACK1 mRNA for 48 hours. Cell lysates were analysed by western blot using antibodies against RACK1, ERK2 and active ERK (pERK). Western blot represents results obtained by at least three independent experiments in which different siRNAs were used.

4.2.2. RACK1 regulates focal adhesion architecture

Since the depletion of RACK1 changes the focal adhesion architecture (Figure 4.1), we quantified length of the focal adhesions. The analysis of the focal

adhesion length showed that RACK1 KD cells formed significantly elongated adhesions (Figure 4.6B). The frequency distribution of the focal adhesions length suggested that RACK1 KD affects all classes of focal adhesions (Figure 4.6C).

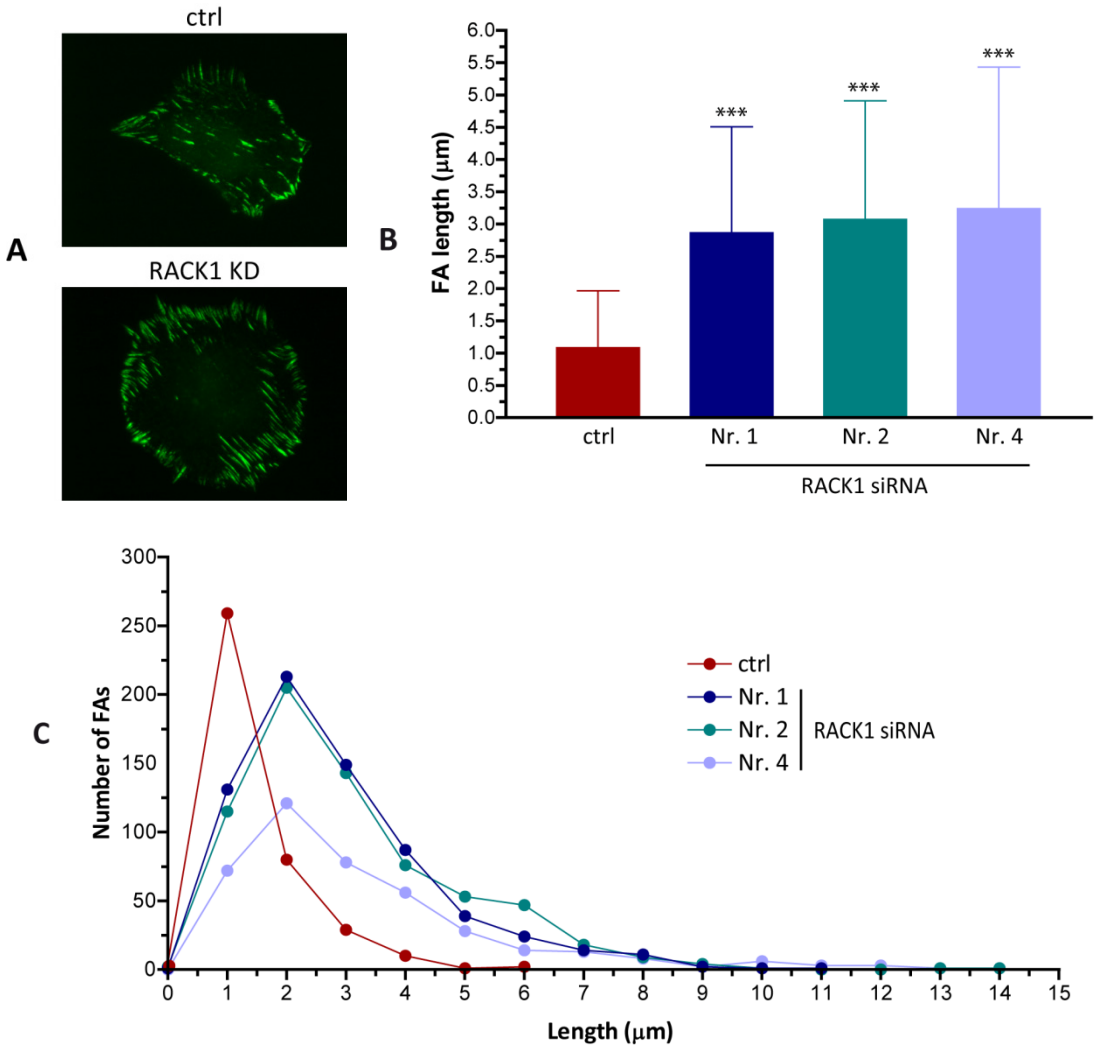

Figure 4.6 - Quantification of focal adhesion length. (A) Control and RACK1-depleted cells stained for paxillin. (B) Quantification of focal adhesion length. The average length of focal adhesions of control cells was approximately 1.1 µm, the RACK1 KD cells produced more elongated focal contacts (approximately 2.8-3.2 µm). (C) The frequency histogram of the focal adhesion length. The number of longer focal adhesions was higher in the RACK1 KD cells. RAT-2 cells were transfected with siRNA targeting RACK1 mRNA for 48 hours and plated on glass coverslips coated with fibronectin (1.0 µg/ml). The Control and RACK1-depleted cells were stained for paxillin. The images obtained by the widefield fluorescence microscopy and the graphs represent data obtained by at least three independent experiments.

4.2.3. RACK1 regulates cell motility during wound healing and random cell migration assay

Since both ERK and RACK1 regulate the focal adhesion turnover and RACK1 is necessary for translocation of active ERK into focal adhesions, it was suggested that ERK and RACK1 control cell migration by regulating focal adhesion disassembly (Vomastek et al., 2007). Indeed, it was shown that RACK1 positively regulates haptotactic migration of REF-52 cells towards fibronectin in Boyden chamber assay (Vomastek et al., 2007). To determine whether RACK1 regulates migration of RAT-2 cells, we performed wound healing assay (Figure 4.7A). We discovered that the control mock-transfected cells recovered the wound more rapidly and efficiently than the RACK1-depleted cells (Figure 4.7B).

Furthermore, we tracked the movement of individual cells migrating towards the wound and measured accumulated distance they travelled and directionality (a value describing how straightforward the movement of particular cell is; the higher value, the more direct trajectory). Both parameters revealed that the RACK1 KD cells, in comparison to the control cells, had impaired migratory abilities as they migrated over shorter distances and the trajectories of their movement were less direct (Figure 4.8). Taken together these data indicate that RACK1 is involved in the regulation of cell migration and that the depletion of RACK1 leads to migration defects.

When we performed random cell migration assay (Figure 4.9), we found that the knockdown of RACK1 did not affect the overall ability of RAT-2 cells to migrate, but disrupted the directionality of their movement. These results were rather surprising as they were contradictory to the results obtained by the wound healing assay.

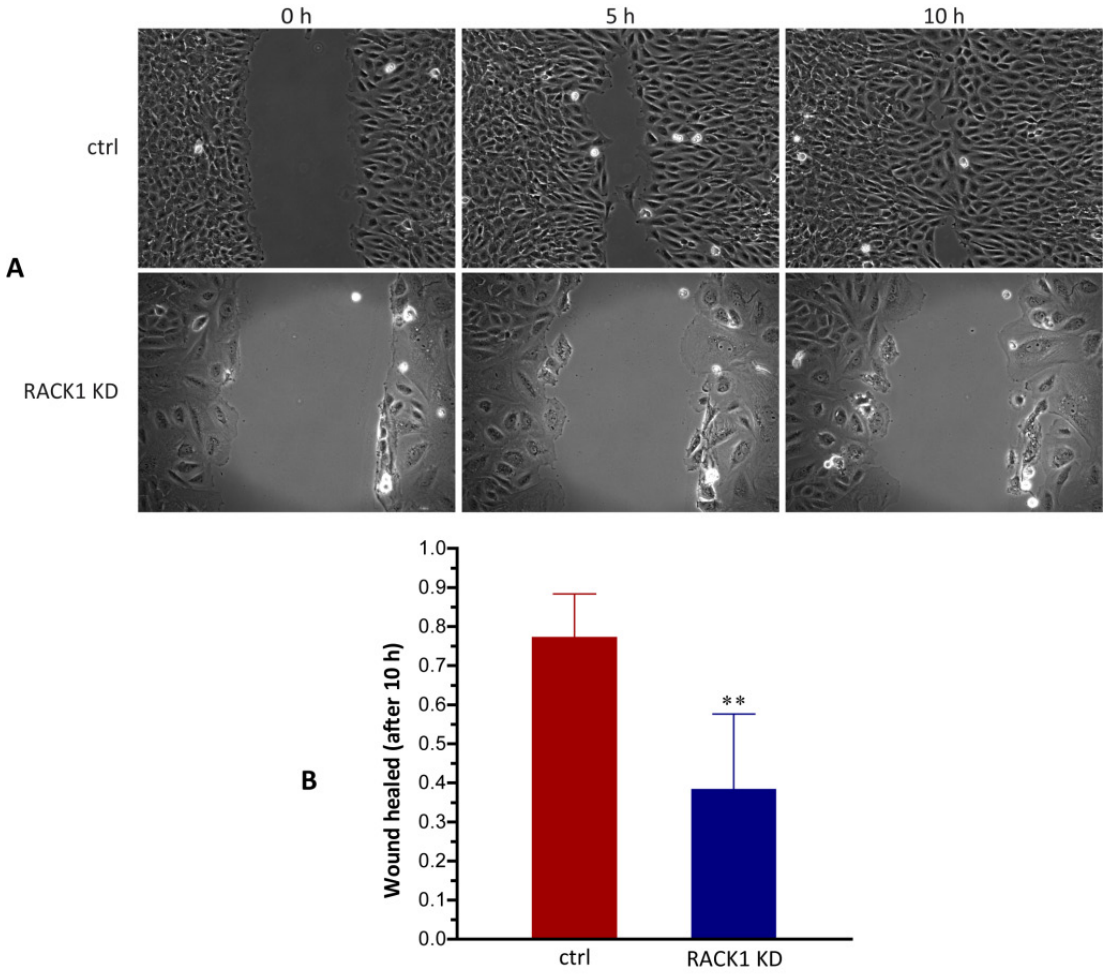

Figure 4.7 – RACK1 regulates cell migration in wound healing assay. (A) RAT-2 cells were transfected with siRNA against RACK1 and 48 hours later a wound was made in a confluent monolayer of cells. Four hours later images were captured every 10 minutes for 10 hours. (B) The silencing of RACK1 significantly impaired the ability of RAT-2 cells to heal the wound. The values in the graph represent portion of the wound healed after 10 hours. The images and the graph represent data obtained by at least three independent experiments.

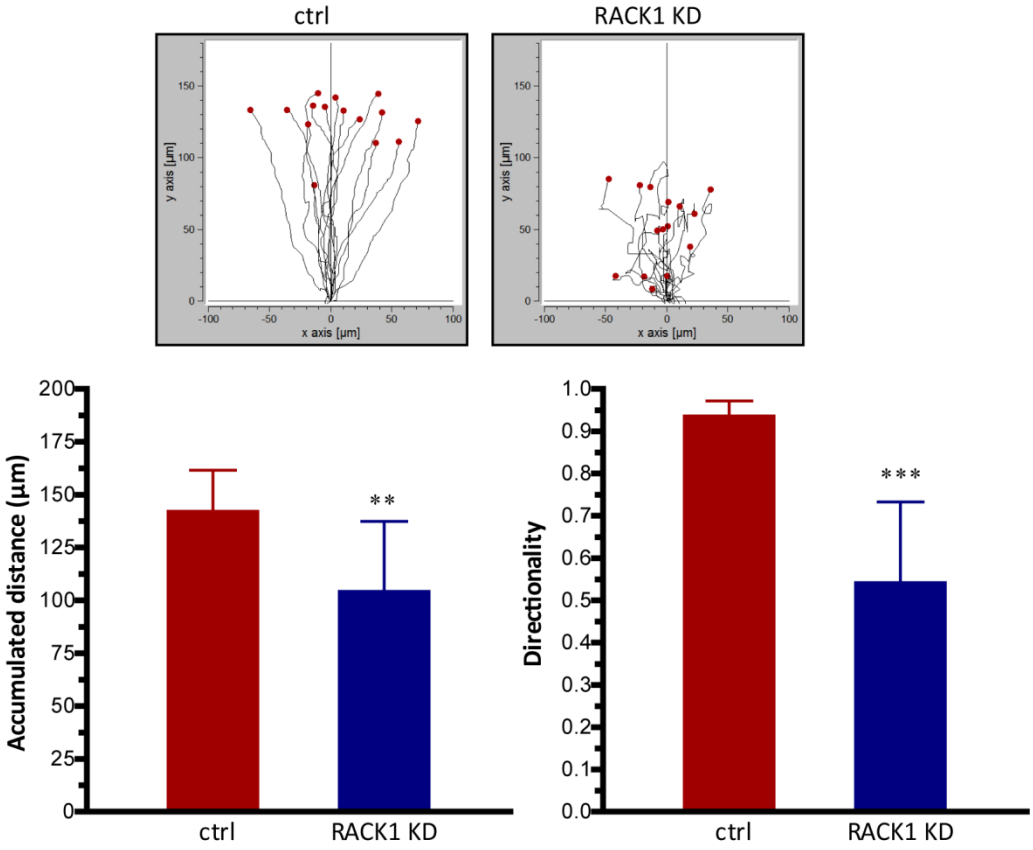

Figure 4.8 – Following the movement of individual cells towards the wound confirmed the requirement for RACK1 during regulation of cell motility. Using Chemotaxis And Migration Tool (Ibidi) we determined trajectories of 15 individual cells and measured two different parameters - accumulated distance and directionality (value describing how straight the trajectory was representing the ratio of the direct distance from start to end point divided by the total track distance; 1 = straight line). The RACK1 KD cells showed significant decrease in both parameters. The images and the graphs represent data obtained by at least three independent experiments.

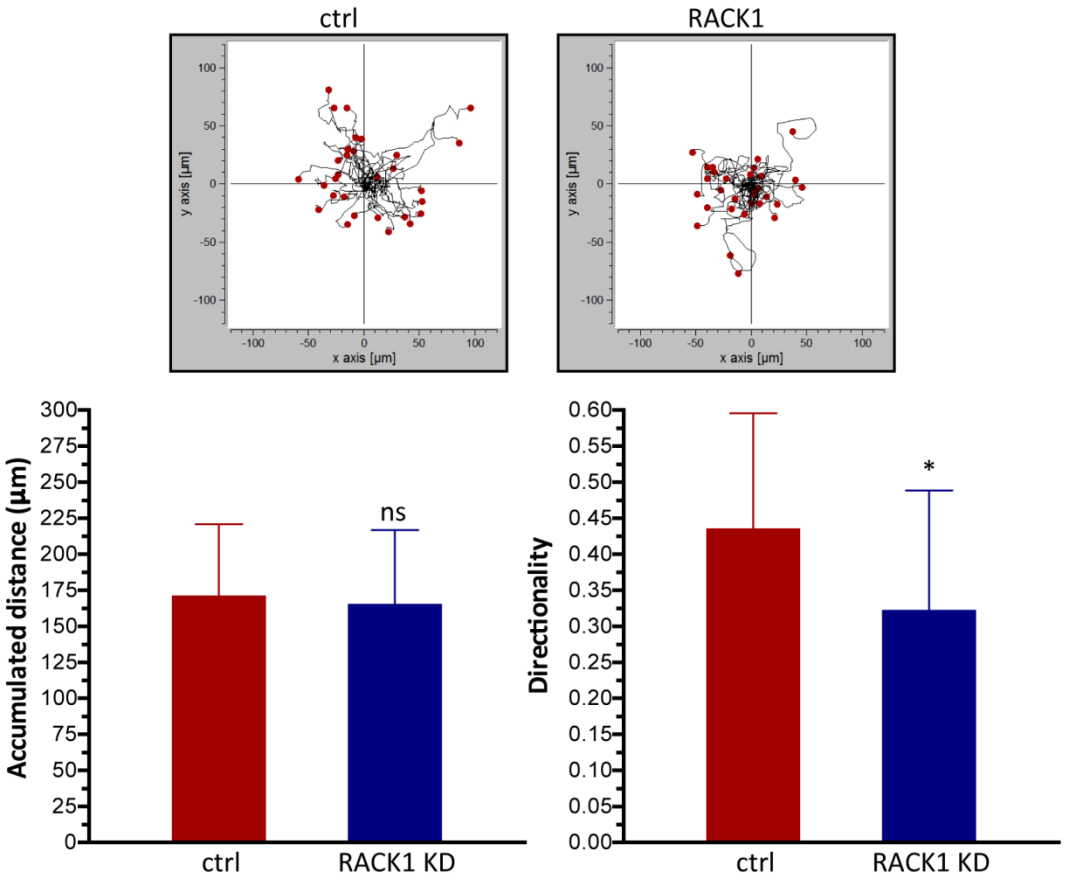

Figure 4.9 – Knockdown of RACK1 affects directionality, but not overall migration ability of RAT-2 fibroblasts during random cell migration. We determined trajectories of 30 individual control and RACK1 KD cells during random cell migration assay by Chemotaxis And Migration Tool. RACK1 depletion led to significant decrease in the directionality of migration, but did not affect the overall ability of RAT-2 to migrate. The images and the graphs represent data obtained by three independent experiments.

4.2.4. RACK1-ERK signalling regulates activity of protein kinase RSK but not phosphorylation of adaptor protein paxillin

Although the contribution of the ERK pathway to the regulation of cell migration has been confirmed by many studies and it is already being widely accepted, the underlying molecular mechanisms still remain unclear. As described in the chapter 2.3.1.2., only few protein targets downstream of ERK that function as migration-specific effectors have been described so far. Identification of novel substrates could provide new information about the regulatory mechanisms utilized downstream of ERK. Moreover, they could help us to untangle the complex and on the same time highly

coordinated signalling network formed by distinct mutually interconnected pathways and to understand the relationship between them. Therefore we decided to look for new downstream targets of the RACK1-ERK signalling module. The hypothetical target should ideally fulfil following criteria: (1) it must be phosphorylated by the protein kinase ERK; (2) this phosphorylation must be RACK1-dependent; (3) it should associate with RACK1; (4) the inhibition of this protein should affect focal adhesions and (5) it must regulate cell migration.

Initially, we compiled a list of potential candidates and selected adaptor protein paxillin. Paxillin was our first choice because it is an integral component of cell adhesions that transmits signals from the FAK-Src complexes and coordinates them with protein machinery responsible for actin cytoskeleton remodelling. Moreover, paxillin was shown to be phosphorylated on Ser83 by protein kinase ERK upon mitogenic stimulation (Ishibe et al., 2004). We examined whether RACK1 silencing induces any significant changes in the paxillin phosphorylation by assessing the level of the Ser83 phosphorylation (p-Ser83; Figure 4.10A). We found that the RACK1-depletion did not affect the phosphorylation of paxillin.

To confirm that the antibody against p-Ser83 in paxillin was functional we investigated the phosphorylation of paxillin upon serum starvation, stimulation by EGF and inhibition of MEK by U0126 (Figure 4.10B). While the serum starvation and inhibition of the ERK signalling led to decrease in the p-Ser83 level, the stimulation by EGF resulted in significant elevation of p-Ser83 suggesting that the phospho-specific antibody worked as anticipated. This result suggested that RACK1 does not mediate the paxillin phosphorylation by ERK and therefore we excluded it from our list of candidate proteins.

Next, we selected protein kinase RSK as the second potential ERK migration-specific target. ERK mediates phosphorylation of several RSK amino acid residues, specifically Thr359, Ser363 and Thr573, a step necessary for activation of RSK (Anjum and Blenis, 2008). Therefore we examined the RSK phosphorylation status upon silencing of RACK1 by assessing the levels of p-Thr359, p-Thr359/p-Ser363 (combined antibody) and p-Thr573 (Figure 4.11A). The analysis of cell lysates revealed that the RACK1 depletion caused reduction of the RSK phosphorylation suggesting that RACK1 contributes to its ERK-mediated activation.

Similarly to the analysis of the paxillin activity, we confirmed the functionality of the antibodies by investigating the RSK activity after serum starvation, stimulation

by EGF and inhibition of MEK by U0126 (Figure 4.11B). The serum starvation and U0126 treatment reduced the levels of all analysed phosphorylated residues and the stimulation by EGF increased the levels of activated RSK indicating that all used antibodies worked appropriately.

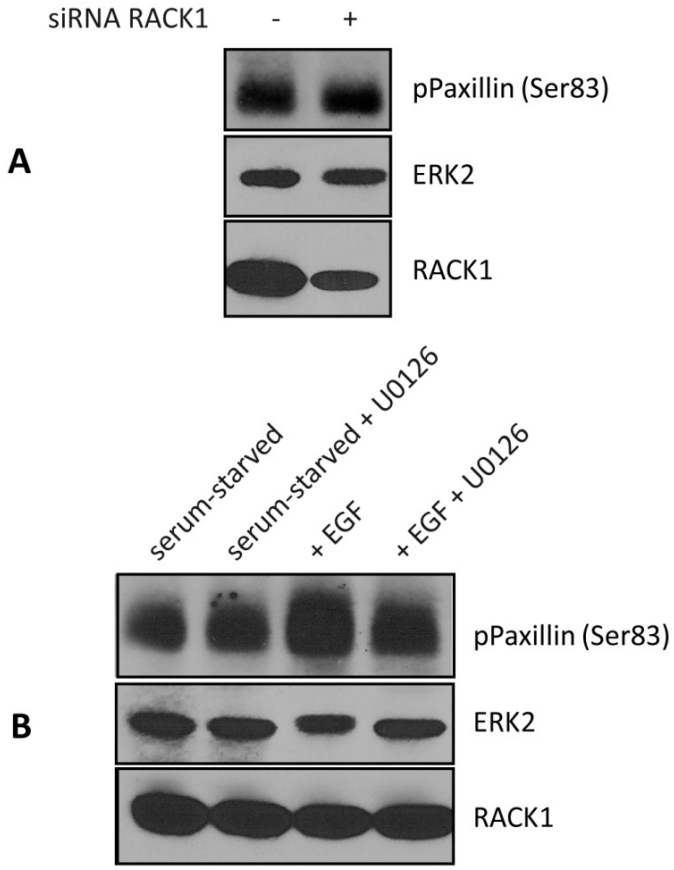

Figure 4.10 – Suppression of RACK1 does not alter the activity of paxillin. (A) RACK1 KD did not change the level of paxillin phosphorylation on Ser83. RAT-2 cells were transfected with siRNA for 48 hours and cell lysates were analysed by western blot using antibodies against phospho-paxillin (Ser83), RACK1 and ERK2. (B) We confirmed that the Ser83-specific antibody works by investigating the activity of paxillin upon serum starvation, stimulation by EGF and inhibition of MEK by U0126. The level of Ser83 phosphorylation increased upon EGF stimulation and declined after the addition of U0126 showing that Ser83 is phosphorylated by ERK.

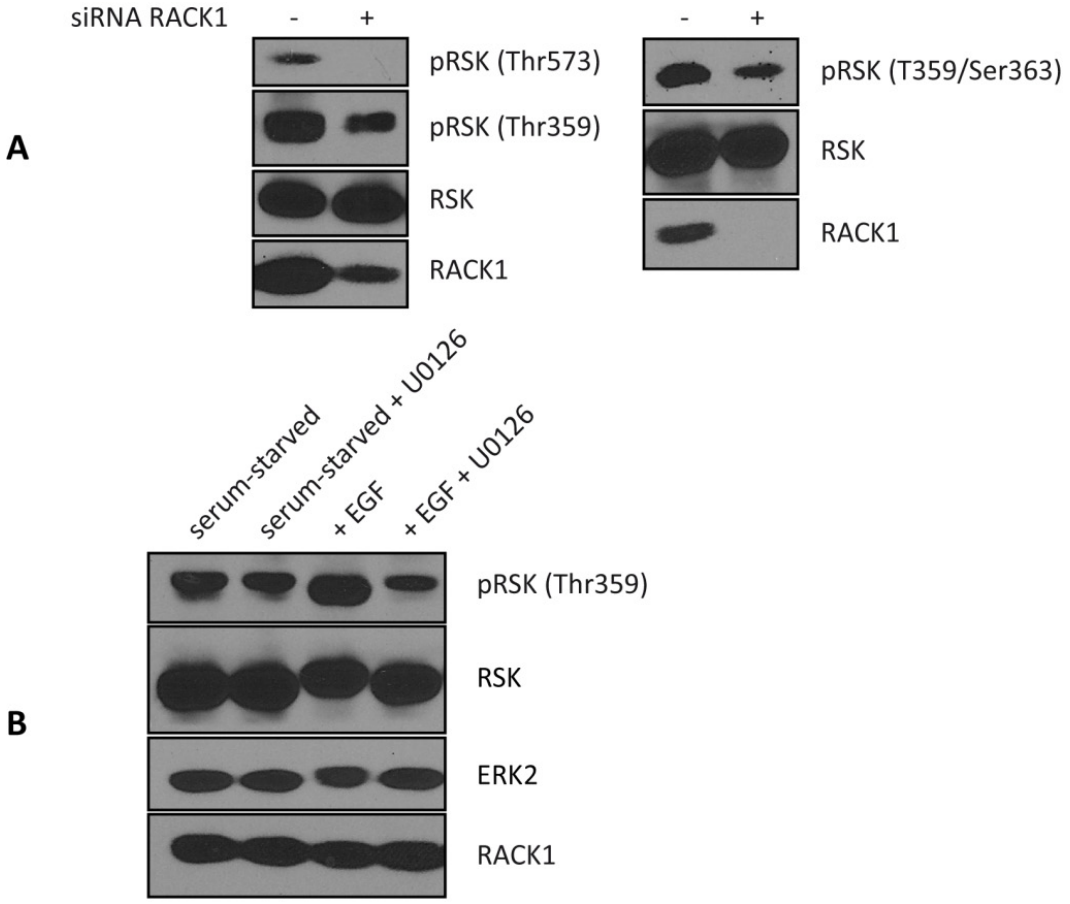

Figure 4.11 – RACK1 regulates activity of protein kinase RSK. (A) Silencing of RACK1 induced reduction of Thr573, Thr359 and Thr359/Ser363 (combined antibody) phosphorylation. RAT-2 cells were transfected with siRNA for 48 hours and harvested. Cell lysates were analysed by western blot using antibodies against phospho-RSK (Thr359; Thr359/Ser363; Thr573), RSK and RACK1. (B) Functionality of the antibodies was verified by exploring the phosphorylation of RSK upon serum starvation, stimulation by EGF and inhibition of MEK by U0126. The serum starvation and application of U0126 decreased the level of RSK phosphorylation, whereas the stimulation by EGF elevated the RSK phosphorylation. To determine the activation status of RSK in response to EGF, cells were maintained in 10% FBS, serum starved for 4 hours and then stimulated with EGF (10 ng/ml) for 30 minutes and harvested. The presented western blot illustrates results obtained by all three phospho-specific antibodies.

4.2.5. RSK forms complex with RACK1

Having found that RACK1 regulates the phosphorylation of protein kinase RSK by ERK, we decided to examine whether this control is achieved through direct interaction of RSK with RACK1. The ability of RACK1 to interact with RSK was tested by co-immunoprecipitation (Figure 4.12). We transiently overexpressed RACK1 (tagged with FLAG epitope) and RSK2 (tagged with HA epitope) in COS-1 cells and then immunoprecipitated FLAG-RACK1 with anti-FLAG antibody crosslinked to agarose beads. Immunocomplexes were resolved by SDS-PAGE and examined for the association of RACK1 with RSK. We found that RSK2 co-immunoprecipitated with FLAG-RACK1 suggesting that RACK1 forms complex with RSK2.

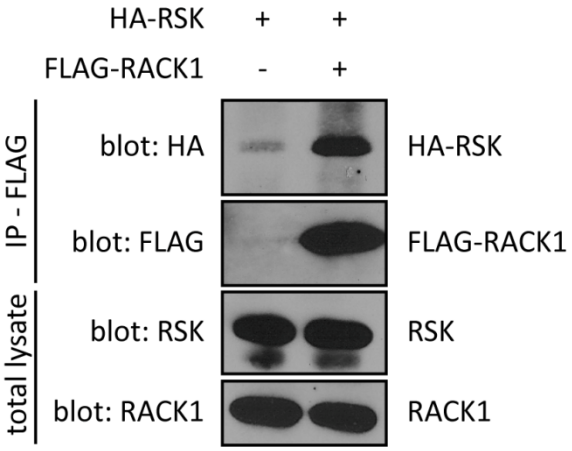

Figure 4.12 - RACK1 associates with RSK. We performed co-immunoprecipitation of RACK1 and RSK from COS-1 cells transfected with FLAG-tagged RACK1 and HA-tagged RSK2. Precipitated RACK1 was detected by FLAG M2 antibody. Co-precipitated RSK2 was detected with HA antibody. Cell lysates were probed with RACK1 and RSK antibodies. The blot represents data obtained by two separate experiments.

4.2.6. Inhibition of RSK phenocopies the knockdown of RACK1

We hypothesized that protein kinase RSK functions downstream of ERK and that RSK requires the presence of RACK1 for its association with ERK and subsequent modulation of cell motility. If our suggestion was correct, then the depletion or inhibition of RSK would trigger phenotypical changes comparable to alterations observed in the RACK1 KD cells. To confirm our hypothesis, we used RSK-specific

pharmacological inhibitor BI-D1870, which acts as an ATP competitive inhibitor of the N-terminal kinase domain of all four RSK isoforms (Sapkota et al., 2007). To show that the inhibition on different levels of the ERK pathway manifests similar phenotype, we blocked in parallel protein kinase MEK by U0126. We treated the RAT-2 cells with the inhibitors for 24 hours and then used wide-field fluorescence microscopy to capture images of individual cells. We found that the BI-D1870- and U0126-treated cells exhibited phenotype similar to the RACK1 KD cells. The inhibition of protein kinase MEK and RSK led to reorganization of actin cytoskeleton and focal adhesion architecture. The overall shape of individual cells also shifted from conical to more circular, a typical feature of RACK1 KD cells, although this effect was not as pronounced as in the RACK1 KD cells (Figure 4.13).

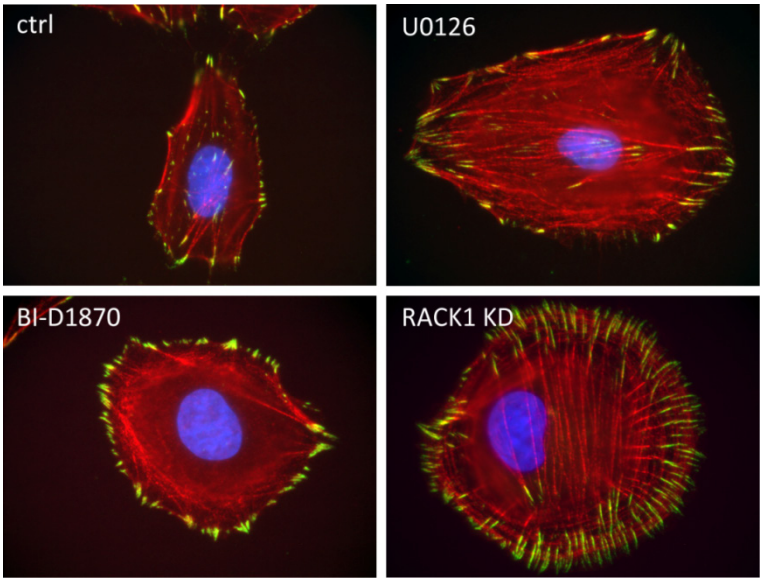

Figure 4.13 – RSK functions downstream of the RACK1-ERK pathway in regulation of actin cytoskeleton and focal adhesion architecture. (A) To explore the effect of inhibition of RSK on cellular phenotype, we blocked the RSK activity by specific inhibitor BI-D1870. To confirm that RSK functions downstream of the RACK1-ERK pathway, we blocked ERK by MEK inhibitor U0126. RACK1 KD cells were prepared by transfection of RAT-2 cells with siRNA for 48 hours. All cells were plated on glass coverslips coated with fibronectin (1.0 µg/ml) and stained with antibody against vinculin and with phalloidin Texas Red-X to visualize actin filaments. Nuclei were stained with DAPI.

The quantification of focal adhesion length indicated that the inhibition of MEK and RSK led to elongation of cell adhesions that was comparable with adhesion length of the RACK1-depleted cells (Figure 4.14). Taken together these data suggested that, similarly to MEK and RACK1, RSK is a component of the signalling cascade utilized during reorganization of actin cytoskeleton and establishment of focal adhesion architecture.

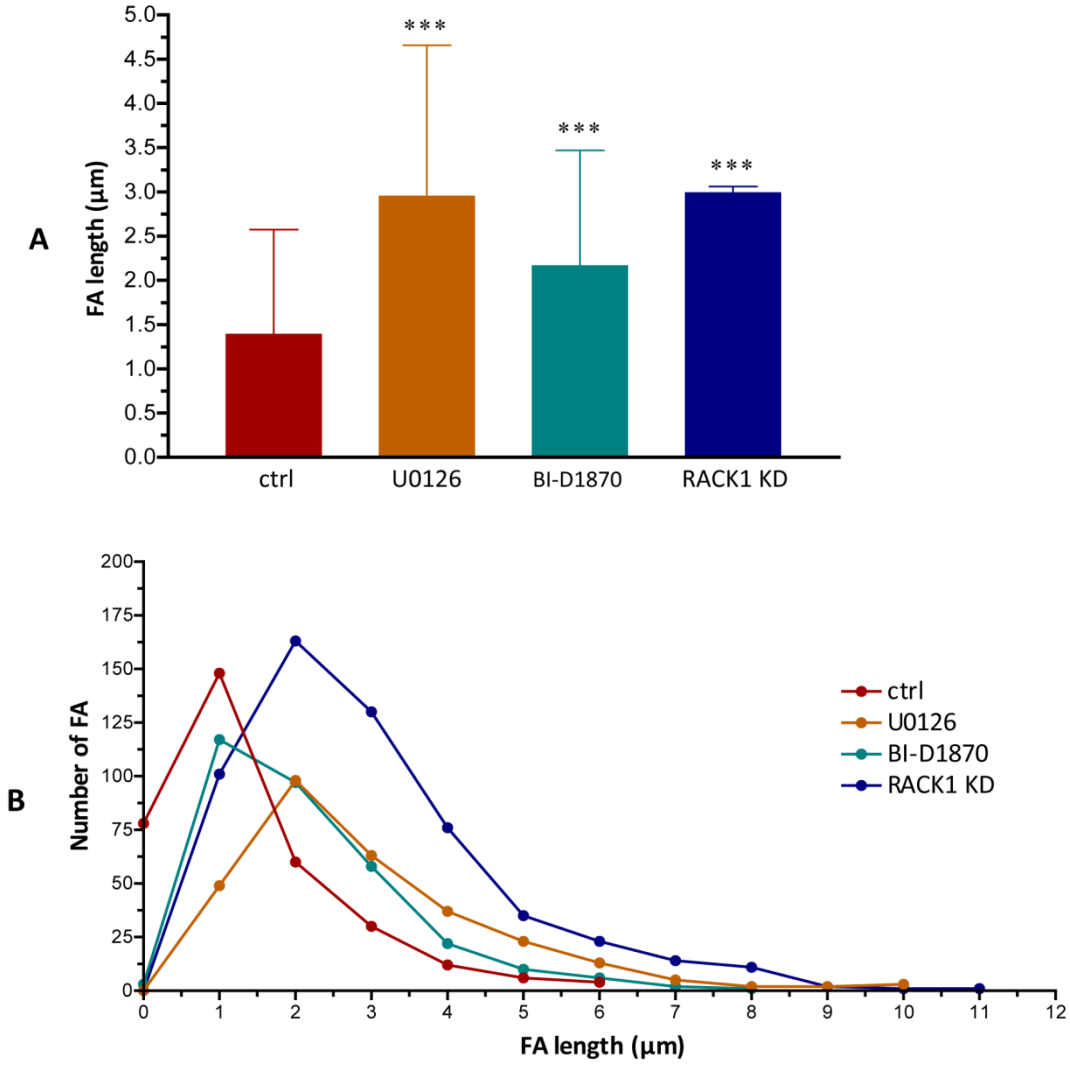

Figure 4.14 - Quantification of focal adhesion length. (A) The inhibition of RSK with BI-D1870, similarly to depletion of RACK1 and MEK inhibition, resulted in significantly longer focal adhesions. (B) As indicated by the frequency histogram, the number of longer focal adhesions was higher in the RACK1 KD and inhibitors-treated cells. The graphs represent data obtained by two independent measurements.

4.2.7. RSK regulates cell motility during wound healing and random cell migration

To test whether RSK participates in the RACK1-ERK mediated regulation of cell motility, we blocked RSK by BI-D1870 and performed wound healing assay (Figure 4.15A). As in the previous experiment, we suppressed MEK by treating RAT-2 cells with U0126. We found that the BI-D1870 treatment significantly impaired the migration of RAT-2 fibroblasts to same level as U0126 (Figure 4.15B). These observations were in agreement with the results obtained when performing wound healing assay with RACK1-silenced RAT-2 cells.

Subsequently, we analyzed cell migration by tracking the movement of individual cells migrating towards the wound and determined the accumulated distance and directionality of each cell. Similarly to the previously obtained data, the U0126- and BI-D1870-treated cells displayed severely decreased migration ability assessed by decline in the accumulated distance (Figure 4.16). However, the BI-D1870 and U0126 treatment did not decrease the directionality as observed upon the RACK1 KD. The U0126-treated cells remained unaffected and the BI-D1870-treated cells even increased the directionality of their movement (Figure 4.16).

In addition to the wound healing assay, we performed random cell migration assay and determined directionality and accumulated distance travelled by individual cells. Although the directionality was not altered significantly, the pharmacological inhibition of MEK and RSK resulted in dramatic decrease in cell motility (Figure 4.17). The results from the random cell migration assay were in acceptance with the data obtained by the wound healing assay, however, they are not entirely consistent with the results from the RACK1 KD cells. Together all these findings indicated that the protein kinase RSK, similarly to MEK, is involved in the regulation of cell migration in RAT-2 fibroblasts.

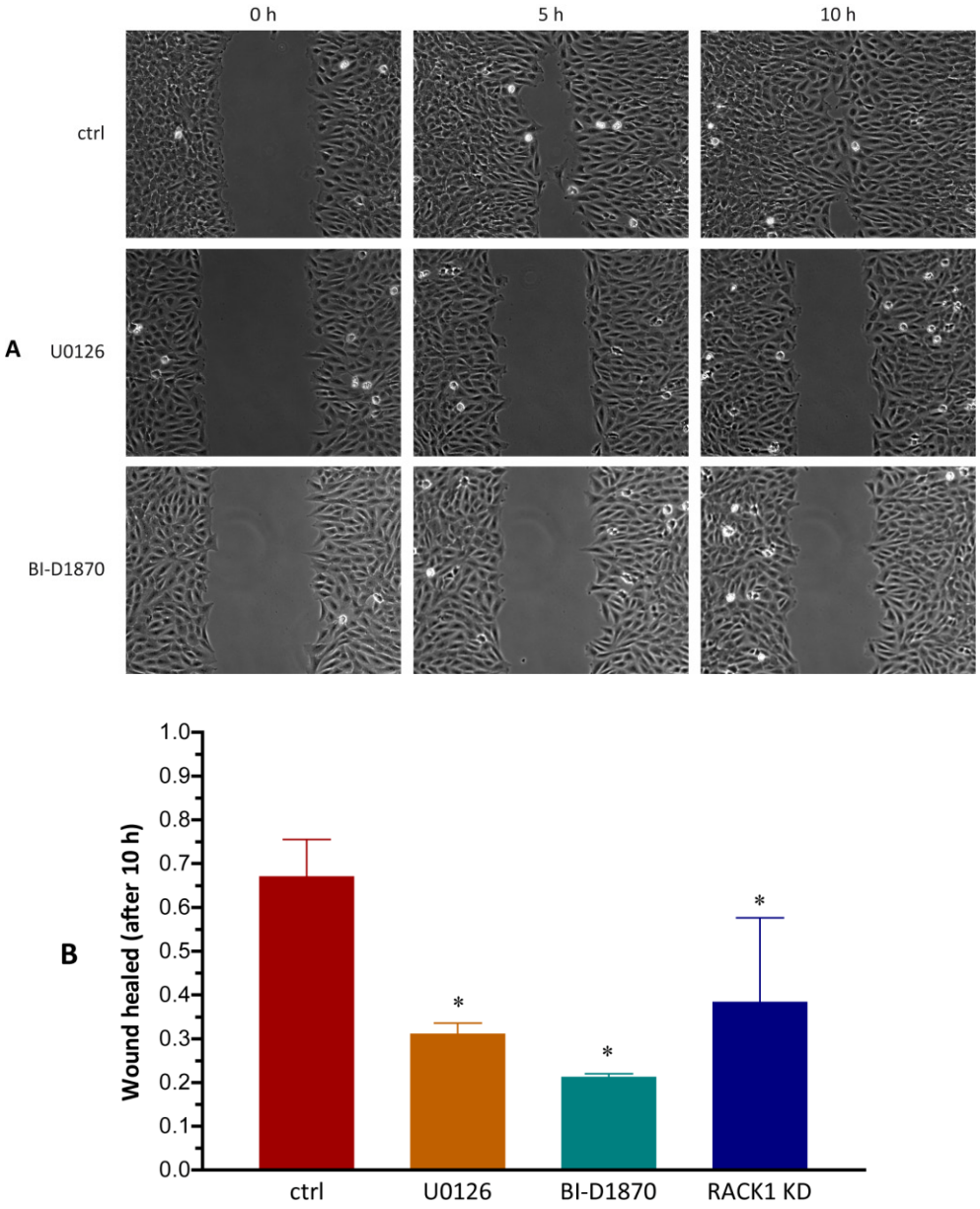

Figure 4.15 – RSK, similarly to RACK1 and ERK, contributes to the regulation of cell migration during wound healing assay. (A) Upon reaching the confluency, a wound was made in a monolayer of RAT-2 cells. Three hours and 30 minutes later inhibitors U0126 and BI-D1870 were added and after additional 30 minutes images were captured every 10 minutes for 10 hours. (B) The inhibition of protein kinase MEK as well as RSK significantly impaired the ability of RAT-2 cells to heal the wound. As indicated, this result was comparable to the effect of the RACK1 suppression observed in the previous experiments. The values in the graph represent portion of the wound healed after 10 hours. The images and the graph represent data obtained by two separate experiments.

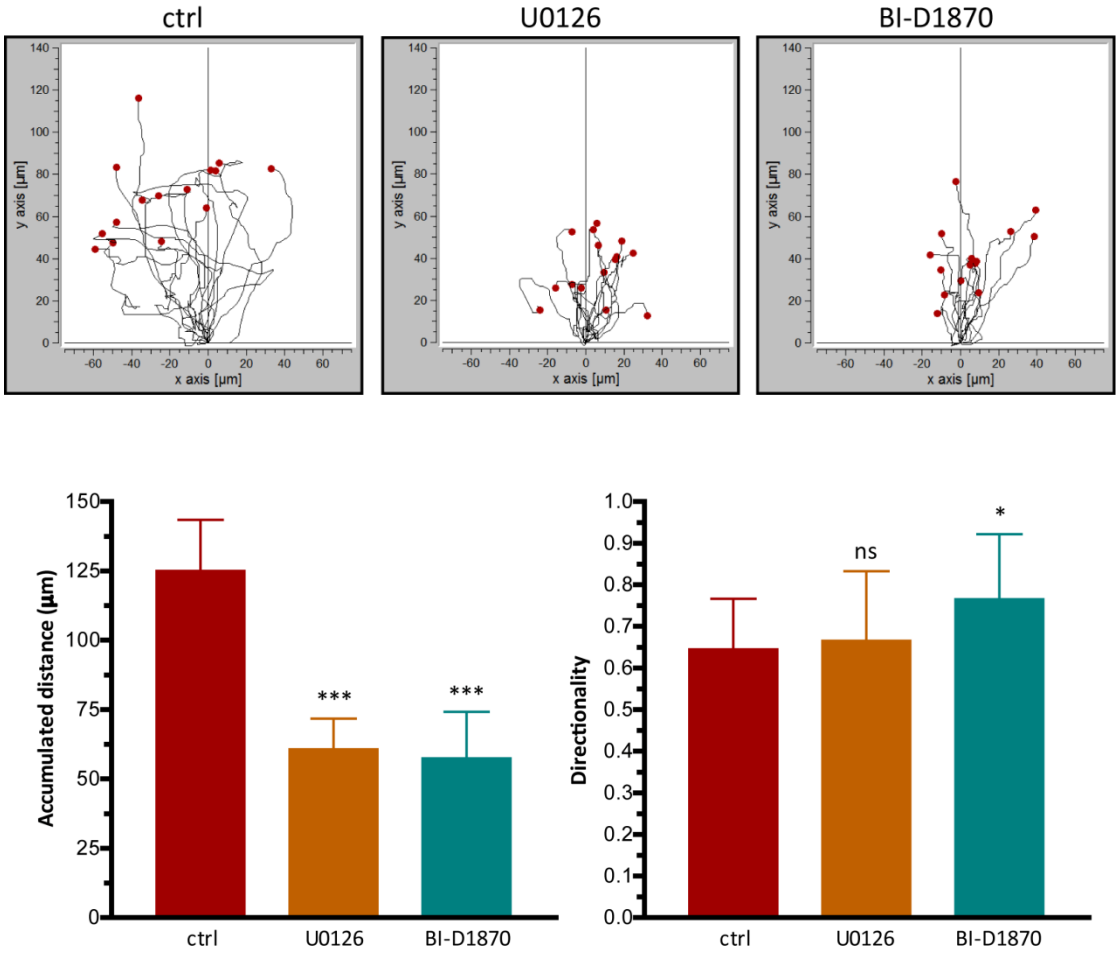

Figure 4.16 – Involvement of RSK in the regulation of cell motility confirmed by tracking the movement of individual cells migrating towards the wound. Using Chemotaxis And Migration Tool software (Ibidi) we determined trajectories of 15 individual BI-D1870- and U0126-treated cells migrating towards the wound (upper panel). Quantification of accumulated distance (lower left panel) revealed that the inhibition of RSK and MEK significantly decreased the motility of migrating cells. Quantification of directionality (lower right panel) revealed that U0126-treated cells remained unaffected and the BI-D1870-treated cells increased the directionality of their movement. The images and the graphs represent data obtained by two independent experiments.

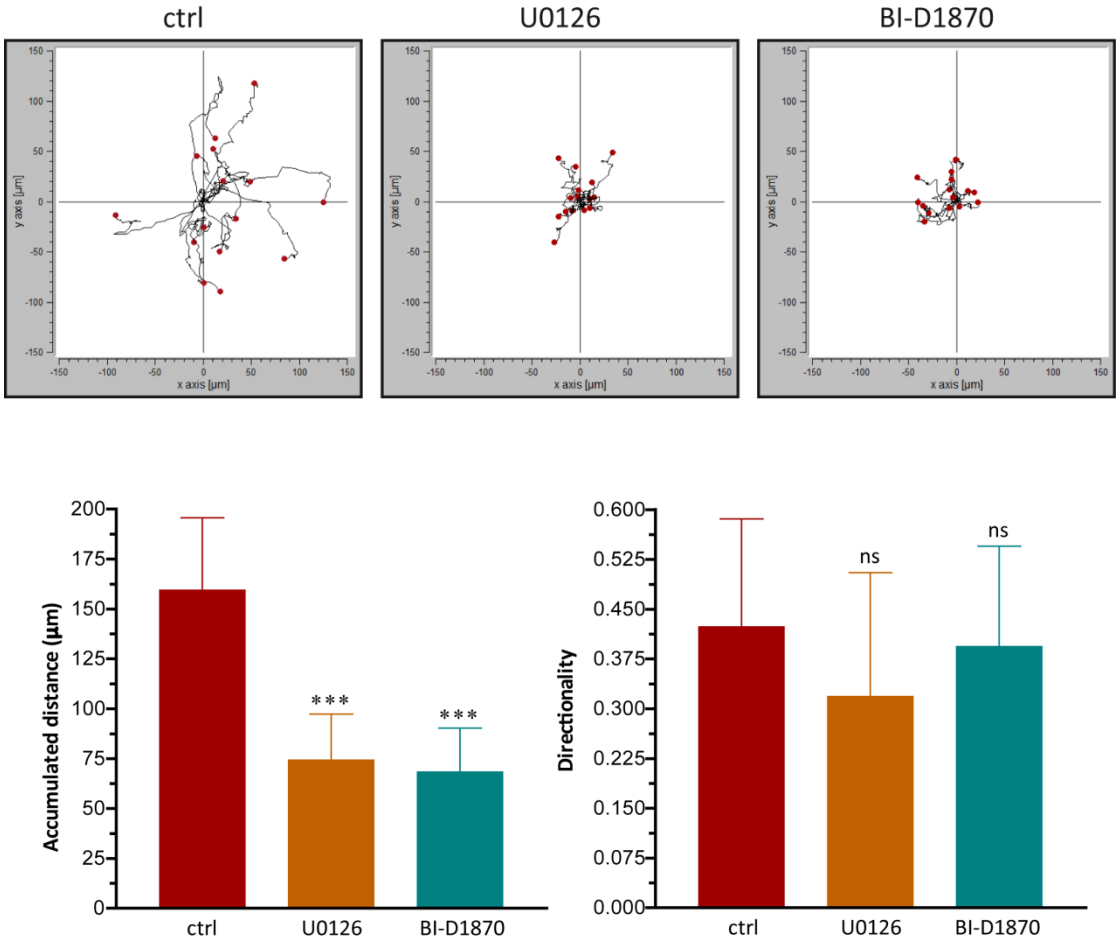

Figure 4.17 – RSK plays role in the regulation of cell motility during random cell migration. We determined the trajectories of 15 individual BI-D1870- and U0126-treated RAT-2 cells during random cell migration assay (upper panel). Quantification of accumulated distance (lower left panel) and directionality (lower right panel) revealed that the inhibition of RSK and MEK significantly decreased the motility but not directionality of randomly migrating RAT-2 cells. The images and the graphs represent data obtained by two independent experiments.

4.2.8. Hyperactivation of the Rho signalling pathway does not mediate the phenotypical changes caused by the silencing of RACK1

We also sought to explore the molecular mechanisms involved in the modulation of cell migration downstream of the protein kinase RSK. Our evidence, the reorganization of actin cytoskeleton, particularly the increase in the number of stress fibres and altered protrusivity upon the RACK1 knockdown, suggested that the changes might be caused by altered activity of Rho GTPases, more specifically by the Rho

signalling pathway. Rho mediates formation of the stress fibres and maturation of focal adhesions through the induction of the actomyosin contractility (Amano et al., 1997). Rho controls activity of multiple substrates including protein kinases MLCK, ROCK, LIMK or actin severing protein cofilin (Figure 2.6) (Raftopoulou and Hall, 2004). Because our data indicated that the RACK1 silencing led to increase in the Rho signalling, we decided to investigate the Rho activity.

First, we directly measured the activity of RhoA, the main Rho isoform, in control and RACK1 KD cells using commercially available kit (G-Lisa Rho assay kit). We detected slight increase in the RhoA activity in the RACK1 KD cells (Figure 4.18). However, technical problems that included the detection of signal from positive control sample made this result unreliable. Moreover, this method suffered from low reproducibility as it strongly depended on many factors (for example confluency of cells).

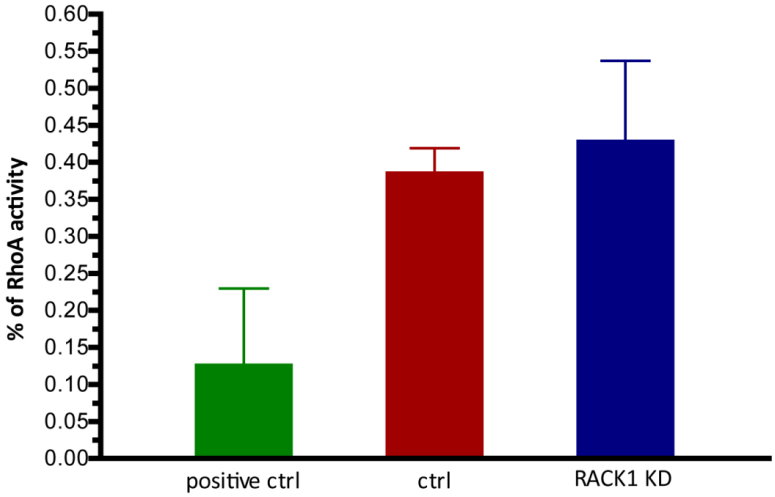

Figure 4.18 – RACK1 KD cells had slightly elevated RhoA activity as measured by G-Lisa RhoA assay. RAT-2 cells were transfected with siRNA against RACK1 and after 48 hours were harvested and processed as described in the manufacturer's protocol. The RACK1-depleted cells exhibited slight increase in the RhoA activity. However, we were not able to measure appropriate signal from positive control sample. The graph represents data obtained by a single experiment performed in duplicates.

Therefore, we investigated indirect evidence for the Rho activity by analysing posttranslational modifications of microtubules. Microtubules (MTs) are dynamic structures that are locally stabilized during cell polarization which is required for

efficient cell migration (Gundersen and Bulinski, 1988). The stabilization is achieved by capping of MT plus-ends to prevent subunit exchange. Stable MTs subsequently undergo posttranslational modifications by detyrosination of α-tubulin. The resulting tubulin is called Glu for the newly exposed glutamate residues. The detyrosinated MTs accumulate additional modifications, acetylations. Palazzo's study demonstrated that the localized stabilization of MTs is achieved by Rho-mDia signalling pathway (Palazzo et al., 2004). We thus used MT detyrosination and acetylation as a readout for the Rho activity. We investigated the level of microtubule detyrosination and acetylation by western blot and found that the RACK1-depleted cells exhibited elevated levels of detyrosinated and acetylated tubulin (Figure 4.19). This was in agreement with our previously suggested hypothesis that the RACK1 KD induces elevation in the Rho activity which is responsible for the phenotypical changes in RAT-2 cells.

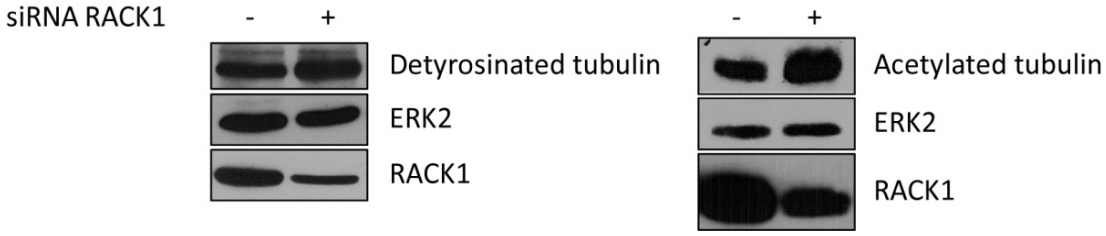

Figure 4.19 – RACK1 KD increases tubulin detyrosination and acetylation, posttranslational marks of stable microtubules. RAT-2 cells were transfected with siRNA against RACK1 for 48 hours and cell lysates were analysed by western blot using antibodies against detyrosinated tubulin, acetylated tubulin, RACK1 and ERK2. Blots represent data obtained by three independent experiments.

We wanted to confirm our hypothesis that the RACK1 KD cells had elevated Rho activity by impairing the Rho signalling by specific inhibitors (Figure 4.20A). Initially, we used the phosphorylation of cofilin as a readout for the Rho activity. Because cofilin is downstream target of Rho-ROCK signalling, the elevated Rho activity should lead to increase in the Ser3 phosphorylation of cofilin (Figure 4.20A). To block the Rho pathway we applied small inhibitory molecule Y-27632 that blocks protein kinase ROCK (Yamaguchi et al., 2006). We analyzed the activity of cofilin in control and RACK1 KD cells and found that the inhibition of ROCK led to decrease in the cofilin phosphorylation as predicted (Figure 4.20B). However, the RACK1 silencing alone did not increase the Ser3 phosphorylation, but rather suppressed it (Figure 4.20B).

This result was in direct conflict with our hypothesis that the RACK1 KD induces the Rho signalling pathway.

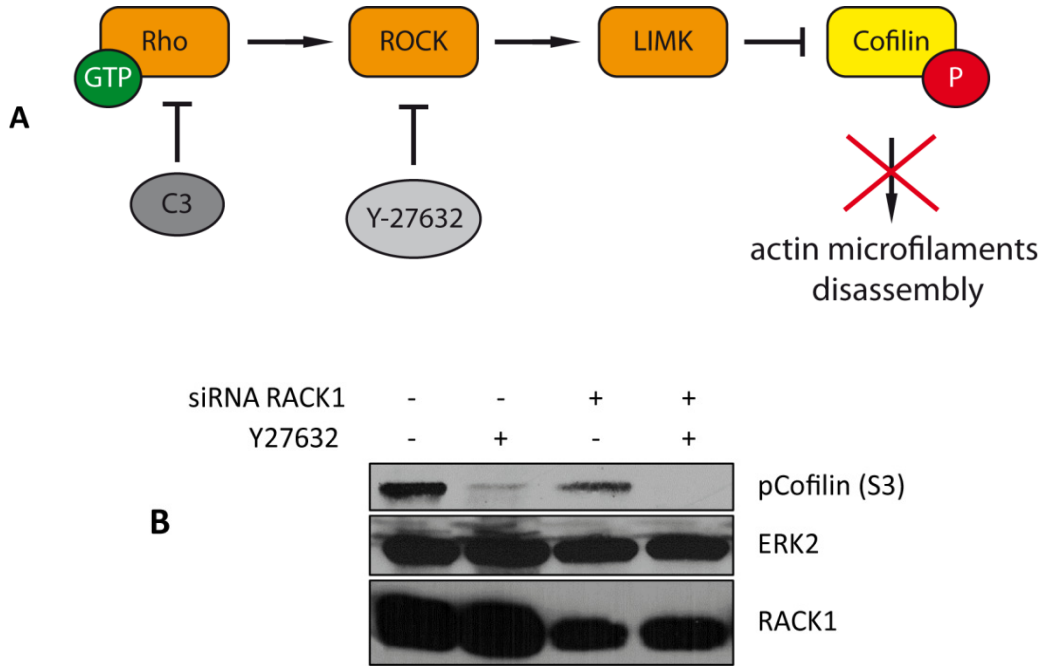

Figure 4.20 - RACK1 KD led to enhanced activity of cofilin. (A) Schematic representation of signalling through Rho. Rho activates protein kinase ROCK that in turn activates LIMK. LIMK is responsible for phosphorylation of cofilin which renders it inactive and prevents depolymerization of actin filaments. (B) Inhibition of ROCK by Y-27632 decreased the level of cofilin phosphorylation. Similar results were obtained after silencing of RACK1 which suggests that RhoA is not responsible for the phenotypical changes observed in the RACK1 KD cells. RAT-2 cells were transfected with siRNA against RACK1 and after 24 hours ROCK-specific Y-27632 inhibitor was added. Control cells were treated with Y-27632 for 24 hours as well. After additional 24 hours both RACK1 KD and control cells were harvested. Cell lysates were analysed by western blot using antibodies against p-cofilin (Ser3), RACK1 and ERK2. The blot represents data obtained by at least independent experiments.

Next, we inhibited total Rho signalling by C3 exoenzyme, an ADP-ribosyltransferase derived from *Clostridium botulinum* (Morii and Narumiya, 1995). We treated control and RACK1 KD cells with the cell permeable C3 transferase and investigated effects on actin cytoskeleton. While in the control cells the C3-treatment resulted in almost complete disassembly of actin filaments, the RACK1 KD cells remained unaffected, without any apparent changes in the actin cytoskeleton architecture (Figure 4.21). These observations confirmed our previous contradictory

result that silencing of RACK1 does not lead to elevation of the Rho signalling. We therefore suggested that the phenotypical changes observed in the RACK1 KD cells are not due to increased activity of the Rho signalling pathway.

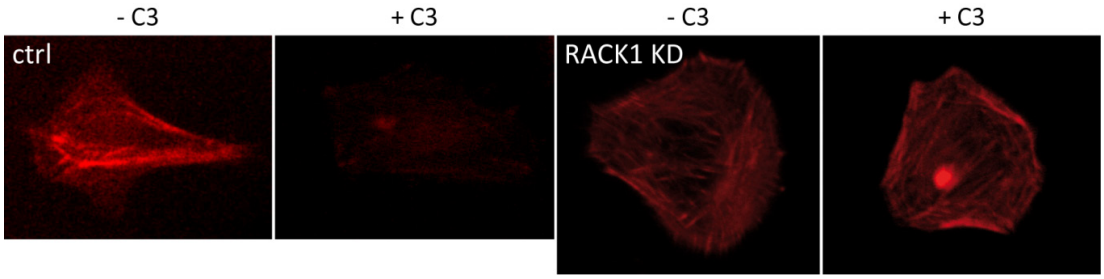

Figure 4.21 – C3 transferase treatment of control and RACK1 KD cells. While treatment of RAT-2 control cells with C3 transferase resulted in almost complete disassembly of actin filaments, RACK1 KD cells remained unaffected. RAT-2 cells were transfected with siRNA targeting RACK1 mRNA for 48 hours and plated on glass coverslips coated with fibronectin (1.0 µg/ml). Afterwards, C3 transferase was added for 4 hours and then the cells were stained with phalloidin Texas Red-X to visualize actin filaments. The images were obtained from a single experiment.

5. Discussion

Taken together, our results suggest that RACK1 is required for efficient regulation of cell protrusivity, cellular shape, adhesion dynamics and cell migration. The analysis of RACK1 function in the regulation of focal adhesion dynamics and cell motility suggests that protein kinase RSK is a principal effector downstream of RACK1-ERK signalling module. We found that RACK1 is required for efficient activation of RSK and that, in addition, RSK forms complex with RACK1. These data strongly suggest that RACK1 is the scaffold protein of the ERK pathway that presents RSK to its upstream activator, ERK. The RACK-mediated activation of RSK specifically channels the ERK signalling towards the regulation of focal adhesions and cell motility. The investigation of underlying molecular mechanisms revealed that the regulatory pathway probably does not directly require the activity of the Rho signalling module.

5.1. RACK1 functions as a signalosome

Initially, the ERK signalling cascade (and generally all signalling cascades) was regarded as linear pipeline that conveys - via its core components, protein kinases Raf, MEK and ERK - signals emanating from plasma membrane and turns them into multitude of cellular responses. However, our understanding of signalling pathways has broaden and we have abandoned this simplistic view because it is turning out that the signalling pathways form highly complex communication networks with many overlapping functions and shared components (Figure 5.1). Moreover, the importance of the core protein kinases in the control of the final outcome had decreased as the contribution of additional proteins (protein kinases or adaptor proteins that mediate crosstalk with other systems) began to be more acknowledged. Now it is evident that the ERK pathway requires interplay between its core protein kinases and various adaptor proteins to ensure an appropriate response upon stimulation by distinct extracellular agents (Kholodenko et al., 2010). It was discovered that it is possible to alter the topology of the ERK pathway and thus reprogram the cellular response solely through adaptor proteins without affecting the input signal. For example, PC-12 cells undergo differentiation instead of proliferation when protein inhibitor RKIP is prevented from

blocking the ERK pathway (Santos et al., 2007). Moreover, it was observed that imbalance in the expression level of the adaptor proteins, rather than the change in the activity of the core signalling components, can contribute to development of for instance endometrial cancer (Martinho et al., 2012), or nasopharyngeal carcinoma (Chen et al., 2009).

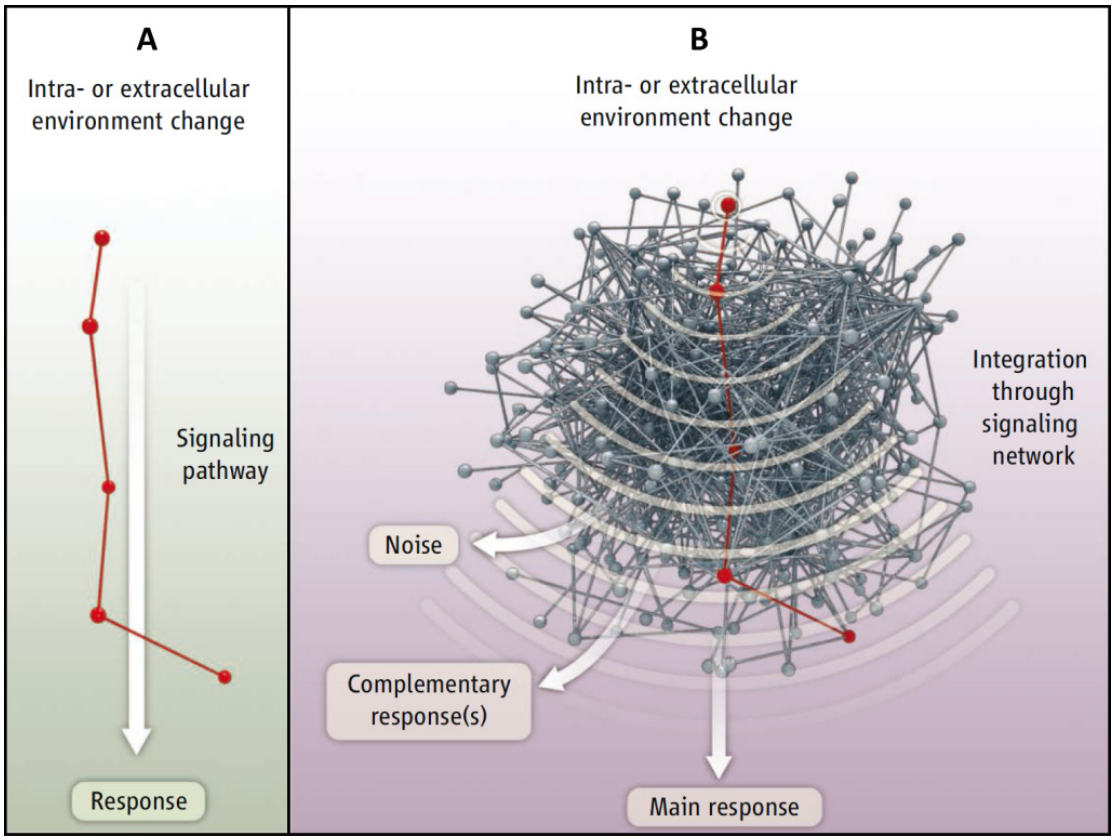

Figure 5.1 – The concept of signalling pathways. Our understanding of the architecture of signalling pathways has changed over time. (A) Initially, the pathways were considered as a linear pipeline composed of few key components. (B) However, many studies showed that despite the importance of the core components of the signalling pathway, additional proteins (components of other signalling cascades or adaptor proteins) can significantly shift the topology of the pathway towards distinct cellular responses. Model by (Levy et al., 2010).

RACK1 is one of the adaptor proteins that contribute to signal transduction through the ERK pathway. However, RACK1 does not operate only at the level of the ERK cascade but rather associates with multiple signalling pathways including PKC, FAK-Src or Akt. In addition, we added the protein kinase RSK as another RACK1-binding protein that functions in the regulation of cell migration. Thereby RACK1

modulates broad range of cellular functions (Adams et al., 2011). We propose that RACK1 forms a multiprotein complex – a signalosome – that enables RACK1 to carry out its regulatory functions at one place and at particular time, thus ensuring a specific cellular response – cell migration (Figure 5.2). The cell migration is a highly coordinated process of significant importance as its aberrant regulation leads for example to impaired immune response or to tumor progression and metastasis. It is evident that understanding the underlying molecular mechanisms is crucial and will help in the struggle with cancer disease. Thus, one of the goals of this study was to explore the RACK1-dependent functions in the regulation of cell migration in RAT-2 fibroblasts.

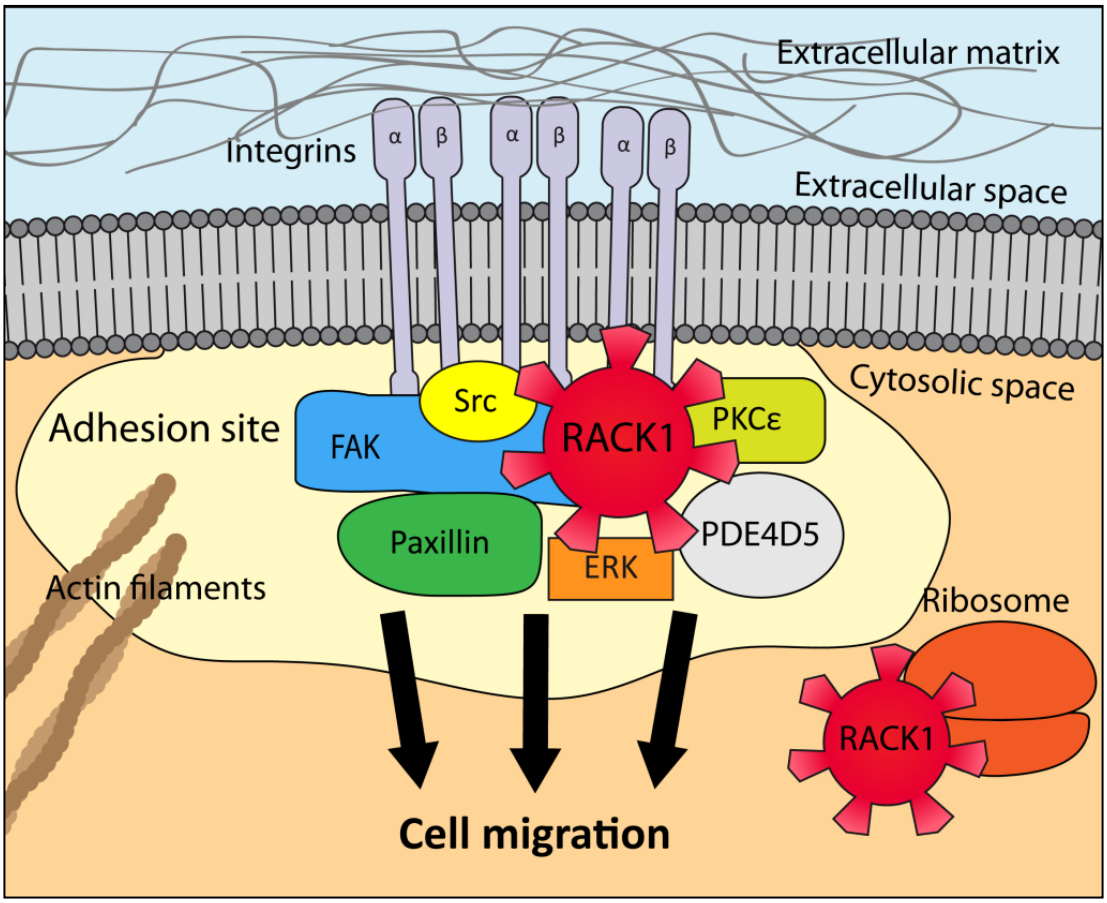

Figure 5.2 – Scaffold protein RACK1 forms a multiprotein complex, a signalosome. RACK1 localizes to cell adhesions and interacts with multiple proteins including FAK, Src, PKC, Paxillin, PDE4D5 or ERK. RACK1 might also recruit ribosomes to cell adhesions and thereby facilitate local protein translation. By coordinating multiple signalling pathways RACK1 regulates cell migration. A modified model adapted from (Adams et al., 2011).

To address this issue, we investigated effects of siRNA-mediated depletion of RACK1 on phenotype of RAT-2 fibroblasts. We found that cells with silenced RACK1 exhibited several typical traits – first of all, significantly altered architecture of actin filaments with more prominent stress fibres; secondly, elongation of focal adhesions and lastly, significant increase in the cell size and shift in the cellular shape from conical (that is typical for RAT-2 fibroblasts) to more circular. These characteristics indicated that RAT-2 cells with suppressed expression of RACK1 might have altered adhesion dynamics and regulation of actin cytoskeleton. We also suggest that the shift in the cellular shape might be caused by compromised ability to efficiently establish leading and trailing edge. This notion is supported by several observations. RACK1-depleted cells displayed reduced ability to form membrane protrusions and reorganized actin cytoskeleton, which is one of the critical factors of the cell polarization. Last but not least, the functional assays showed that RACK1 indeed regulates directional migration.

However, to confirm that RACK1 regulates the cytoskeleton protrusivity and directional migration more precise analysis (for example measurement of velocity of membrane protrusions) would be necessary. Unfortunately, we currently do not have a functional and reliable tool that would allow us to objectively quantify the protrusivity in the peripheral area of entire cells.

5.2. The role of RACK1 in focal adhesion disassembly

Despite the great deal of attention that has been paid to untangle the chain of migration-modulating events downstream of ERK, only few potential targets of ERK have been discovered to date. The organization of focal adhesion architecture indicated that RACK1 regulates focal adhesion dynamics and pointed towards the ERK signalling cascade and its deregulation. We thus focused on dissecting the underlying molecular mechanisms to gain an insight into the involvement of RACK1 in the ERK-mediated regulation of the focal adhesion disassembly.

RACK1 associates with all three core protein kinases of the ERK signalling module - Raf, MEK and ERK - and links them to upstream activators and various downstream effectors. Moreover, RACK1 regulates ERK localization into focal adhesions (Vomastek et al., 2007) and it was predicted that in focal adhesion RACK1 facilitates activation of effector proteins that subsequently regulate cell motility by inducing the focal adhesion disassembly.

Thus, we have focused on finding new potential effector proteins utilized by the RACK1-ERK module during the regulation of cell motility and we identified protein kinase RSK, a novel binding partner of RACK1. We also tested an adaptor protein paxillin but excluded it from the list of the RACK1-binding partners as the RACK1 KD did not significantly alter the phosphorylation of paxillin by ERK. Inhibition of protein kinase RSK led to phenotypical changes similar to the alterations observed upon the silencing of RACK1 or the inhibition of protein kinase MEK – i.e. elongation of focal adhesions and reorganization of actin cytoskeleton. However, to gain more information, it would be necessary to design a RSK-specific siRNA. More precisely, to design siRNAs against all three major isoforms to eliminate the effect of functional redundancy they commonly share. This would also allow us to determine the contribution of individual RSK isoforms.

These data suggested that RSK is a direct downstream effector of the ERK module targeted by RACK1. RACK1 associates with integrins, FAK, Src, components of the ERK pathway and RSK. It is tempting to hypothesise that RACK1 serves as a "signalling channel" that links the integrins and the FAK-Src signalling to and presents it to RSK downstream targets, thereby promoting a coordinated focal adhesion disassembly and cell motility. We can support our hypothesis by evidence confirming that RACK1 (Cox et al., 2003), ERK (Fincham et al., 2000) and RSK (Schiller et al., 2011) are all localized to focal adhesions. On the basis of the available evidence we proposed model of the RACK1-ERK-RSK signalling (Figure 5.3).

However, at the present time we cannot conclusively confirm the RACK1 and RSK involvement in the focal adhesion disassembly as all our measurements of the adhesion dynamics were performed indirectly, based on the adhesion length rather than on assembly/disassembly rates. We could not measure focal adhesion dynamics as we unfortunately were not able to establish a functional model that would allow us to determine directly the rate of the focal adhesion disassembly. We tried to transiently transfect RAT-2 cells with siRNA against RACK1 and with GFP-paxillin but the cells did not cope with this combination very well as they underwent apoptosis. Also the efficiency of double transfections is very low and makes it hard to perform experiments requiring high number of cells. We were also unsuccessful when preparing cell line stably expressing GFP-paxillin. The direct measurement of the adhesion dynamics will be the subject of our future studies as it could help us to understand which pathways might be involved in the process.

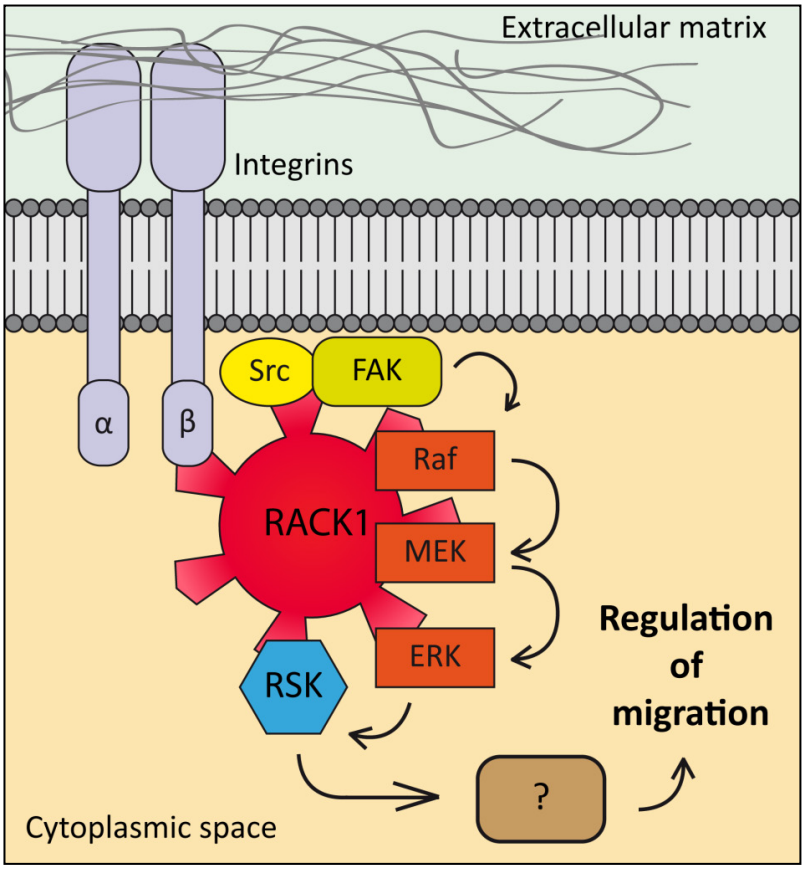

Figure 5.3 – Schematic model of signalling through the RACK1-ERK-RSK pathway. RACK1 functions as a signalling platform facilitating the contact of the core protein kinases of the ERK pathway. RACK1 links the whole ERK signalling module to its upstream activators, integrins and FAK-Src complexes. At the same time RACK1 presents the ERK pathway to its downstream effectors such as protein kinase RSK to modulate a specific cellular response such as cell migration. RSK acts through unknown downstream effectors.

We investigated events further downstream of the protein kinase RSK and the RACK1-ERK pathway. Our data implied that the RACK1-ERK-RSK cascade might negatively affect the activity of Rho signalling pathway and that the absence of one the signalling components might result in the Rho hyperactivation. The Rho pathway is responsible for formation of the actin stress fibres and for regulation of events at the cell rear that lead to maturation and subsequent disassembly of cell adhesions. Therefore the hyperactivation of the Rho pathway results in defects such as impaired adhesion disassembly and increased formation of actin stress fibres, typical features of the RACK1 KD cells or cells with inhibited protein kinase RSK and MEK. However, the investigation of indirect markers of the Rho hyperactivation did not support our

hypothesis that the Rho signalling pathway directly mediates the reorganization of actin cytoskeleton and other phenotypical changes observed in the RAT-2 cells upon silencing of RACK1. Although our evidence excludes the hyperactivation of the Rho signalling upon the impairment of the RACK1-ERK-RSK cascade, we cannot rule out the possibility that the pathways function in parallel or via some alternative signalling components. This notion is supported by the finding that RhoA might be a RSK-dependent regulator of cell migration (Smolen et al., 2010).

The signalling events downstream of RACK1 and the protein kinase RSK remains unknown although the available literature points to specific RSK effectors that might be involved in the regulation of cell migration. In genome-wide RNAi screen several proteins that contribute to cell motility downstream of protein kinase RSK were identified (Smolen et al., 2010). These include coronin 1A, p21Cip1, p27Kip1, SH3P2, Rnd3, VASP (vasodilator-stimulated phosphoprotein) and Filamin A.

Coronins are group of proteins that interact with Arp2/3, cofilin and other regulatory proteins and contribute to control of actin filament structure at the leading edge of the cell (Humphries et al., 2002). Coronin 1A, a member of the coronin family, was shown to be required for translocation of Rac1 to plasma membrane which leads to enhanced local activity of Rac1 (Castro-Castro et al., 2011). Coronin 1A was identified as a potential downstream migration-specific target of the protein kinase RSK (Smolen et al., 2010) and it is thereby possible that coronin 1A participates in the same signalling pathway as ERK and RACK1.

Second candidate target of RSK suggested by the Smolen's study is p21Cip1 (Smolen et al., 2010). p21Cip1 is well described protein inhibitor and target of p53 that blocks multiple cyclin-CDK complexes and mediates DNA damage-induced cell cycle arrest in G1 and G2 phase (Besson et al., 2008). However, it has been shown that cytoplasmic pool of p21Cip1 binds to and inhibits ROCK1, which results in the inactivation of Rho signalling and decrease in cofilin phosphorylation (Lee and Helfman, 2004). Thereby, p21Cip1 contributes to the regulation of cell motility and it would be interesting to explore whether the cytoplasmic fraction of p21Cip1 requires efficient RACK1-ERK-RSK signalling.

p27Kip1 is another inhibitory protein that regulates cell cycle progression by interaction with cyclin-CDK complexes. Similarly to p21Cip1, cytoplasmic pool of p27Kip1 plays additional role in other cellular processes. p27Kip1 regulates cell migration in fibroblasts as $p27Kip1^{-/-}$ cells formed higher number of actin stress fibres

and focal adhesions due to increased activity of RhoA. It was suggested that p27Kip1 binds to RhoA and inhibits its activation by GEFs (Besson et al., 2004). The activity of p27Kip1 is regulated by Akt and RSK-mediated phosphorylation which leads to association with 14-3-3 and to subsequent cytosolic relocalization (Fujita et al., 2003). If we consider that the cytosolic fraction of p27Kip1 has a migration-specific function, the RSK/Akt-mediated phosphorylation might provide an interesting mechanism how to control cell motility.

Functional screening of HeLa S3 cell cDNA library revealed a novel RSK substrate, SH3P2 (Tanimura et al., 2011). SH3P2 was originally isolated as an SH3 domain-containing protein of unknown function (Sparks et al., 1996). Later, it was discovered that SH3P2 is a negative regulator of cell motility. The ability to block the migration is prevented by RSK1-mediated phosphorylation. Because SH3P2 localizes to focal adhesions, it was suggested that SH3P2 might serve as a multifunctional adaptor protein contributing to the cell migration (Tanimura et al., 2011).

Rnd3 is another potential effector of RSK. Rnd3 is a member of a Rho GTPases family and functions as a Rho antagonist. It was shown that Raf activation induces the expression of Rnd3 and that the expression precedes a loss of actin stress fibres suggesting that Rnd3 is involved downstream of the ERK pathway (Hansen et al., 2000). Another study identified Rnd3 as an activator of p190RhoGAP, which is responsible for inactivation of RhoA (Wennerberg et al., 2003). These data indicated that Rnd3 might function as a link between the ERK and Rho signalling pathway. This was confirmed by recent study showing that Rnd3 mediates crosstalk between the ERK module and Rho/ROCK pathway and therefore contributes to the control of actin cytoskeleton and focal adhesion architecture (Klein et al., 2008).

Other RSK candidate targets include protein VASP and filamin A. VASP is an actin-binding regulator that antagonizes capping of actin filaments. RSK1 interacts with VASP and phosphorylates it on Thr278 (Lara et al., 2011), a residue that is required for efficient binding of actin (Benz et al., 2009). Filamin A was amongst the first identified migration-specific targets of RSK. After mitogen stimulation, RSK associates with Filamin A and phosphorylates it on Ser2152 (Woo et al., 2004). The phosphorylation of Ser2152 is required for binding of PAK1 and for subsequent control of actin cytoskeleton architecture and membrane ruffle formation (Vadlamudi et al., 2002). These data indicate the existence of alternative pathways utilized by RSK and possibly also by the RACK1-ERK cascade during the regulation of cell motility.

5.3. RACK1, ERK and RSK in the regulation of cell migration

Although our study brought answers to many of the initial issues concerning the regulation of focal adhesion disassembly and cell migration by the ERK signalling pathway and the scaffold protein RACK1, it also raised several new questions. The greatest controversy arose from exploration of the effects of RACK1 on cell migration. We found that despite the apparently impaired focal adhesion disassembly, the RACK1-depleted cells did not show any significant change in their migration abilities during random cell migration. This was rather surprising because we expected that the rate of the adhesion disassembly might be the limiting step in the cell migration process. We also discovered that the inhibition of RSK and ERK, in contrast to silencing of RACK1, did not lead to significantly altered directionality of the cellular movement during both wound healing and random cell migration. The discrepancy in our results might be in part caused by preliminary nature of the data presented here. We have for example observed movement of quite a limited number of cells (15-30 from one sample in one experiment) and therefore we will have to confirm our results by analysing significantly larger group of cells. Moreover, in contrast to the treatment with inhibitors, which were added 30 minutes before imaging, the knockdown of RACK1 lasts at least 48 hours and therefore it might have induced more pronounced effects on intracellular organization of actin and focal adhesions. On the other hand, the silencing of RACK1 and the subsequent impairment in the adhesion disassembly might really not be the limiting factor in the cell motility because similar behaviour was observed in RACK1-depleted REF-52 cells migrating towards EGF in Boyden chamber (T. Vomastek, personal communication). It is tempting to speculate that unaffected, control cells form dynamic adhesions that are predominantly localized at the cell front within the leading edge, thus establishing and maintaining polarized morphology and ensuring the persistent directional movement. On the other hand, the RACK1 KD cells may form dynamic adhesions in a non-polarized manner. This inability to establish and maintain the cell polarity might consequently result in the loss of directional movement. Additional experiments involving live time imaging of focal adhesion dynamics will be necessary to resolve this issue.

In conclusion, we speculate that RACK1 as an adaptor protein communicating through several signalling cascades might convey its regulatory functions by multiple alternative routes. Although our results strongly imply that RACK1, ERK and RSK are

involved in the process of cell motility, it remains to be proved whether ERK, RSK and RACK1 contribute to the cell motility by signalling through common pathway or rather act in parallel by some alternative mechanism. We will address this question in prospective experiments.

6. Summary

- Silencing of RACK1 protein in RAT-2 fibroblasts leads to dramatic changes in cell morphology and induces remodelling of actin cytoskeleton and focal adhesion architecture.
- Using kymographs we show that RACK1 regulates cell protrusivity.
- RACK1 regulates adhesion-induced, but not serum-induced activation of ERK
- RACK1 protein regulates cell motility during wound healing and random cell migration.
- RACK1-ERK signalling is required for regulation of protein kinase RSK. The activity of adaptor protein paxillin is RACK1-ERK-independent
- RSK forms complex with RACK1
- Inhibition of RSK phenocopies the knockdown of RACK1
- RSK together with RACK1 and the ERK pathway regulates cell motility
- Hyperactivation of the Rho signalling pathway does not mediate the phenotypical changes caused by the silencing of RACK1

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